Studies on the development of certain fungal and bacterial biopesticides for the management of wilt disease complex of chickpea caused by *Fusarium* and *Meloidogyne* species

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CERTIFICATE

This is to certify that **Mr. F. A. Mohiddin** has worked as a research scholar in the Department of Plant Protection, Faculty of Agricultural Sciences, Aligarh Muslim University, under my supervision and guidance. His work on **"Studies on the development of certain fungal and bacterial biopesticides for the management of wilt disease complex of chickpea caused by *Fusarium* and *Meloidogyne* species"** is original and upto-date. He is allowed to submit this thesis for consideration of the award of the degree of **Doctor of Philosophy (Agriculture) Plant Protection.**

6 August, 2007

Mujeebur Rahman Khan

TO

Bhoba

May her soul rest in a best place in heaven (Aameen)

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(F. A. Mohiddin)

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INTRODUCTION

With the introduction of high yielding cultivars, agricultural productivity in India has not increased to the level expected as the new crop cultivars have failed to give yield to the genetic level because of several reasons. Plant diseases caused by various pathogens is one of the important constraints in obtaining adequate yield of cultivars. Soil-borne fungi, bacteria and nematodes are highly destructive pathogens and cause tremendous yield losses to all kinds of crops. During the last several decades, numerous phytopathogenic fungi, *Pythium*, *Phytopthora*, *Botrytis*, *Rhizoctonia*, *Fusarium* etc. and nematodes, *Meloidogyne*, *Heterodera*, *Radopholus* etc. have spread to new areas especially in developing and underdeveloped countries because of lapses in the implementation of domestic quarantine measures. The exotic pathogens have established better than the native organisms and have become rather more aggressive causing severe damage at pre-harvest as well as post-harvest stage of a crop (Chet *et al.*, 1997).

Wilt caused by *Fusarium oxysporum* f. sp. *ciceri* and root-knot caused by *Meloidogyne incognita* and *M. javanica* are important diseases of chickpea and occur almost in every chickpea growing areas in India. Annual chickpea yield losses from the wilt vary from 10-15% (Jalali and Chand, 1992; Trapero-Casas and Jimenez-Diaz, 1985), but the disease can destroy the crop completely under specific conditions or may cause much greater losses (Navas-Cortes *et al.*, 2000). At severe infection level, the disease may inflict 5.2 q/ha yield loss (Khan, 2005). Root-knot nematodes (*M. incognita* and *M. javanica*) have been reported to cause 19-40% economic loss to chickpea in India (Ali *et al.*, 2003). Ali (1997) reported 25-60% avoidable loss due to *M. incognita*. Besides direct damage, root-knot nematodes possess great capability to synergise other soil-borne pathogens leading to development of disease complex (Khan, 1993). The wilt disease complex caused by the species of *Fusarium* and *Meloidogyne* is a commonest and devastating disease and is considered an important constraint in the production of grain pulses in India (Khan and Reddy, 1993). Infection by *Meloidogyne* spp. not only increase wilt severity but also moderate resistance of a cultivar (Atkinson, 1892; Reed and Lateef, 1990; Castillo *et al.*, 2003). In India many field tolerant cultivars of chickpea

viz., Avrodhi, ICC 12275, ICC 11322, ICC 11319 and ICC 12272 have become susceptible to *Fusarium* in the presence of root-knot nematodes (Ali *et al.*, 2003).

With the advent of chemical compounds it was thought that a permanent and reliable solution of plant diseases have been achieved but it was realized soon that pesticide application is not safe to the environment as the toxicants cause soil pollution and have harmful effects on human beings. Unfortunately, to control a target organism with a pesticide, over 100 species of non-target organisms are adversely affected (Tjamos *et al.*, 1992). However, pesticides are not always injurious, if are applied at lower doses, the application cause less adverse effects. Hence to reduce the dose of chemicals, one possibility is to utilize the disease suppressing activity of certain microorganisms, which should be highly antagonistic against the targeted pathogens; should possess prerequisite biosafety standard and their mass production should be cost effective. Such microorganisms are commonly referred to as biological control (biocontrol) agents and their commercial formulations as biopesticides. However, it is not necessary that a biopesticide should always contain live microorganisms, the formulation may also have microbial metabolites.

Recent researches on biological control have been conducted with greater systemic approach and practical utility. Various microorganisms viz., fungi, bacteria, mycorrhizae etc. have been tested for their ability to suppress plant diseases. As most of the soil-borne plant pathogens are fungi, biocontrol by fungi has been attempted extensively (Henis *et al.*, 1979; Baker, 1987; Suarez *et al.*, 2004). Most of the studies, however, have used strains of *Trichoderma* species (Benitez *et al.*, 2004; Federico *et al.*, 2007). In addition, *Bacillus subtilis* and *Pseudomonas fluorescens* have also been widely evaluated for suppression of plant pathogens (Idris *et al.*, 2007; Someya *et al.*, 2006; Kenneth *et al.*, 2006).

The genus *Trichoderma* comprises numerous fungal strains that act as biocontrol agents to antagonize plant pathogenic fungi in particular. The antagonism by these fungi involves multiple mechanisms. *Trichoderma* strains compete with the pathogens for nutrients and space, modify the microenvironment, produce plant growth promoting substances, elicit plant defensive mechanisms and produce antibiotics, or directly parasitize the pathogenic fungus and cause hyphal lysis. These direct and indirect mechanisms may operate together during the disease suppression. Effectiveness of *Trichoderma* strains against a pathogen depends on several factors

such as the target fungus, plant species and environmental conditions, including soil nutrition, pH, temperature and iron concentration. Activation of each mechanism implies the production of specific compounds and metabolites such as plant growth factors, hydrolytic enzymes, siderophores, antibiotics and carbon and nitrogen permeases.

Among *Trichoderma* species *T. harzianum, T. virens* and *T. viride* are most common and important biocontrol agents. Application of *T. harzianum* T35 suppressed *F. oxysporum* by competing both for rhizosphere colonization and nutrients, with biocontrol becoming more effective when nutrient concentration decreased (Tjamos *et al.*, 1992). Wilt of chickpea caused by *F. oxysporum* f. sp. *ciceri* was effectively managed with the application of *T. harzianum* both *in vitro* and under field conditions (Singh *et al.*, 2003). Soil application of *T. harzianum* resulted to a lowest wilt incidence in chickpea (4-5%) compared to the control (16-17%) (Prasad *et al.*, 2002). *T. harzianum* has better antagonistic efficiency against 10 isolates of *F. oxysporum f. sp. ciceri* compared to *T. viride* (Gurha, 2001). Khan *et al.* (2004) observed a decrease of 59% in the wilt severity of chickpea caused by *F. oxysporum* f. sp. *ciceri* with *T. harzianum*. Application of *T. harzianum* formulation based on tea leaves and wheat bran sawdust significantly reduced mortality in chickpea and groundnut due to wilt complex and collar rot disease, respectively. The CFU load of *T. harzianum* after 30 days was maximum in tea leaves (8 x 108 cfu/g) and shelf life was found to be maximum (2.9 x 105 cfu/g after 210 days) in wheat bran sawdust (Singh *et al.*, 2007). *T. harzianum* was found antagonistic to *R. solani* and *V. dahliae* and inhibited the development of *R. solani* and *V. dahliae in vitro* (Santamarina and Rosella, 2006). Among various bacterial and fungal isolates, *T. virens* isolates GL3 and GL21 provided the most effective suppression of damping-off caused by *R. solani* in greenhouse bioassays. These isolates also provided the most consistent and effective suppression of damping-off of cucumber caused by *Pythium ultimum* (Daniel *et al.*, 2005).

Bacillus spp. are important group of bacteria which are omnipresent in soils and possess the characteristics of high thermal tolerance, rapid growth in liquid culture, formation of resistant spores and broad spectrum activity of their antibiotics. The most important species of the bacteria is *Bacillus subtilis*, which is considered as an efficient biocontrol agent (Shoda, 2000; Cavaglieri *et al.*, 2005). The strain CE1 of *B. subtilis* was highly antagonistic against maize root colonization of

Fusarium verticillioides (Cavaglieri *et al.*, 2005). *Bacillus subtilis* 'E' was found effective in inhibiting fungal growth of *F. oxysporum* f. sp. *ciceri in vitro* (Dikkar *et al.*, 2003). Rangeshwaran *et al.* (2002) reported that *B. subtilis* (PDBCEN 3) was antagonistic to *F. oxysporum* f. sp. *ciceri* and provided (38%) inhibition in a dual culture test. Seed bacterization with *Bacillus* spp. reduced the number of wilted chickpea plants in sick plots infested with *F. oxysporum* f. sp. *ciceri* (Kumar, 1996). Dhedhi *et al.* (1990) reported two isolates of *B. subtilis* antagonistic to *F. oxysporum* f. sp. *ciceri in vitro*. Seed treatment of pigeonpea with *B. subtilis* effectively controlled pigeonpea wilt and enhanced the yield considerably (Nakkeeran and Renukadevi, 1997). Application of Serenade, a commercial biofungicide formulation of *B. subtilis*, to the stigmatic surface of open blue berry flowers suppresses floral infection by the mummy berry fungus *Monilinia vaccinii-corymbosi* (Ngugi *et al.*, 2005). *Bacillus* species have exhibited a high inhibition towards mycelial growth of *Fusarium oxysporum* f. sp. *albedinis* (70-77%) and its sporulation (80-95%) as compared to control (Hassni *et al.*, 2007). Increasing numbers of *Bacillus* strains have been found to be effective in the control of foliar diseases, including gray molds (Lee *et al.*, 2006; Collins and Jacobson, 2003; Hang *et al.*, 2005; Paul *et al.*, 1998; Toure *et al.*, 2004; Gueldner *et al.*, 1988).

Fluorescent pseudomonads are another active and dominant group of bacteria in the rhizosphere and have been extensively investigated for its biocontrol potential (Fernando *et al.*, 2007; Someya *et al.*, 2006; Kishore *et al.*, 2005; Ahmadzadeh and Sherifi-Tehrani, 2006; Nautiyal *et al.*, 2002). Among them, *P. fluorescens*, *P. putida* and *P. cepacia* have been widely researched for practical applications (Shoda, 2000). Inamul Haq *et al.* (2003) evaluated different strains of *P. fluorescens* for the biological control of chickpea wilt and found isolate no. 1, 2, 4, 5 and 9 to be highly effective against wilt fungus and reduced the disease incidence by 92-96% over control. Selected isolates of *P. fluorescens* (Pf1-94, Pf4-92, Pf12-94, Pf151-94 and Pf179-94) induced systemic resistance in chickpea against Fusarium wilt and significantly reduced the wilt disease by 26-50% as compared to control (Saikia *et al.*, 2003). Pande and Chaube (2003) observed a reduction of 18-100% in rice sheath blight infection due to pretreatment of sclerotia for 4 weeks with suspension of *P. fluorescens*. Leeman *et al.* (1995a) demonstrated that *P. fluorescens* could induce systemic resistance in radish against *Fusarium* whereas the bacteria can also trigger a part of systemic acquired resistance against the fungus (Hoffland *et al.*, 1995).

Khan *et al.* (2004) have recorded 39% yield with the seed treatment of *P. fluorescens* as compared with the untreated control and wilt incidence decreased to 25-27%. Bacterization of chickpea seeds with a siderophore producing fluorescent pseudomonad reduced the number of chickpea wilted plants in a wilt sick soil by 52% (Kumar and Dubey, 1992). *Pseudomonas fluorescens* LRB3W1 inhibited the growth of *Fusarium oxysporum* f. sp. *conglutinans* causing cabbage yellows (Someya, 2007).

The nematophagous fungus, *Pochonia chlamydosporia* (= *Verticillium chlamydosporium*) is a potential biocontrol agent against root-knot and cyst nematodes (Kerry, 1993; Atkins *et al.*, 2004) and parasitizes the eggs/adult females of plant parasitic nematodes (Kerry, 2000). All stages of the fungus (hyphae, conidia, chlamydospores) occur in soil and actively growing mycelium infects eggs and females (Davies *et al.*, 1991). The fungus forms close contact with the eggshell and causes disintegration of eggshell, vitelline layer and partial dissolution of chitin and lipid layer (Lopez Llorca and Duncan, 1988; Saifullah and Thomas1997; Stirling, 1991). Egg hatching in the presence of fungus may be inhibited probably due to toxins secreted by the fungus (Meyer *et al.*, 1990; Morgan Jones *et al.*, 1983). Khan *et al.* (2002 and 2005b) have demonstrated that application of *P. chlamydosporia* through root-dip or seed treatment effectively controlled the root-knot of greengram and chickpea and significantly improved the yield of infected plants. De Leij *et al.* (1993) have recorded 95% decrease in the soil population of *M. hapla* due to *P. chlamydosporia* treatment. Some studies have also demonstrated antagonistic effect of *P. chlamydosporia* towards plant pathogenic fungi such as *F. oxysporum* and *R. solani* (Ehteshamul *et al.*, 1994; Jacobs *et al.*, 2003). Hence *P. chlamydosporia* can suppress the diseases caused by fungi and plant parasitic nematodes.

Trichoderma, *Bacillus* and *Pseudomonas* species can also antagonize plant parasitic nematodes in addition to fungal plant pathogens (Pant and Pandey, 2002; Sikora, 1988; Khan *et al.*, 2005a,b). Hatching of *M. incognita* eggs was significantly reduced after incubation with pure PRAI preparations from *T. harzianum* CECT 2413 (Suarez *et al.*, 2004). Liquid culture of *B. subtilis* at a concentration of 0.1-0.6% were highly toxic to juveniles of *M. incognita*, *R. reniformis* and *Tylenchulus semipenetrans* (El-Sherif *et al.*, 1995). Siddiqui and Shaukat (2004) have recorded 28 and 25% decrease in the soil population of root-knot nematode,

Meloidogyne spp. in tomato plants applied with *T. harzianum* and *P. fluorescens,* respectively compared to the inoculated control. Exposure of root-knot nematode to culture filtrates of *P. fluorescens* significantly reduced egg hatch and caused substantial mortality to *M. javanica* juveniles (Siddiqui and Shaukat, 2003). Meyer *et al.* (2001) have found that the number of eggs and second stage juveniles of *M. incognita* per gram root of bell pepper were significantly lower following combined application of *Pseudomonas* isolates and *T. virens* than the untreated control. Other studies have also shown the antagonistic effect of *Bacillus* spp. and *Pseudomonas* spp. on *Meloidogyne* spp. (Kerry, 2000; Giannakou *et al.*, 2007; Padgham and Sikora, 2007; Siddiqui and Shaukat, 2003; Khan *et al.*, 2005a,b).

Many private and governmental agencies are involved in the commercial production of biopesticides and are marketing the products with different trade names (Singhal, 2004). Out of numerous materials tested for mass production of *T. harzianum* and *P. chlamydosporia*, leaf litter, compost and sawdust were found to support greater population of the biocontrol agents (Khan *et al.*, 2001). Tiwari *et al.* (2004) evaluated grains of sorghum, wheat, pearl millet, wheat bran, rice bran, organic manures, cellulosic wastes and sugarcane bagasse for mass multiplication of *T. harzianum* and *T. viride*. Oat kernels, oat seeds, kaolin or wheat bran were used successfully to produce commercial formulation of *P. chlamydosporia* (Kerry *et al.*, 1984; Godoy *et al.*, 1983; Rodriquez- Kabana *et al.*, 1984). Anita and Rajendran (2002) used talc powder for mass multiplication of *P. fluorescens*. Formulation of *B. subtilis* AF1 has been prepared in peat supplemented with either 0.5% chitin or *Aspergillus niger* mycelium or in spent compost obtained from *Agaricus bisporus*. Shelf life of *B. subtilis* in soybean flour lasted upto 3 months (Lewis *et al.*, 1995).

Critical analysis of the relevant researches and the above evidences have revealed that *Bacillus subtilis, Pseudomonas fluorescens, Pochonia chlamydosporia, Trichoderma harzianum* and *T. virens* possess great capability of being potential biocontrol agents and effectively suppress soil-borne plant pathogens such as *Fusarium* and *Meloidogyne* species. Application of these biocontrol agents may enhance the crop yield leading to greater economic return to the growers. But a systemic and practical approach is needed to develop efficient formulations which could yield higher CFU load of these biocontrol agents. The formulations presently available in India contain a low CFU load, which further decreases over time because the substrate hardly support survival of the organisms.

If some new and efficient immobilizing substrates are explored and developed which could support the survival as well as multiplication of the microorganisms during storage and marketing, the formulation will yield much greater CFU load and consequently a better management of the disease will be expected. The present study was undertaken with an objective to develop suitable formulations of biocontrol agents to achieve an effective and satisfactory control of wilt (*Fusarium oxysporum* f. sp. *ciceri*), root-knot (*Meloidogyne incognita*) and the resulting wilt disease complex of chickpea (*F. oxysporum* f. sp. *ciceri* and *M. incognita* concomitantly). To attain the objective following studies were undertaken:

1. Isolation and characterization of local strains of *Trichoderma harzianum, T. virens, Pochonia chlamydosporia, Bacillus subtilis* and *Pseudomonas fluorescens,* and *in vitro* evaluation for antagonism against *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita.*

2. Screening of various industrial and agricultural materials to mass culture biocontrol bacteria and fungi (crude formulations).

3. Evaluation for relative effectiveness of crude formulations of *T. harzianum, T. virens, P. chlamydosporia, B. subtilis* and *P. fluorescens* against wilt (*F. oxysporum* f. sp. *ciceri*), root-knot nematode (*M. incognita*) and wilt disease complex of chickpea (*F. oxysporum* f. sp. *ciceri* + *M. incognita* concomitantly) under pot culture.

4. Screening of different materials for immobilization of selected biocontrol agents (based on performance; Expt. 3) to produce their biopesticides and to test shelf life of the products.

5. Field evaluation of the biopesticides developed for effectiveness against wilt, root-knot and disease complex of chickpea.

REVIEW OF LITERATURE

Pulses are widely cultivated in India especially by small holding farmers. They are low input crops because of their ability to assimilate atmospheric nitrogen, which contribute to the fertility of the soil and also enhance productivity of non-leguminous crops in the rotation. Equally important is their role in the diet of the people particularly the rural people who cannot afford expensive animal products to meet their dietary needs of essential amino acids (Table 1). The pulses efficiently complement the cereal rich food in making a wholesome meal by balancing the amino acid and micronutrient content of the diet. No wonder they are called 'poor mans meat'.

Presently, pulses are grown on around 68 million hectares with 57 million tonnes of production worldwide. In India, latest estimates for 2005-06 indicate that the production of pulses in the country is 13.4 million tonnes from an area of 22.4 million hectares (Anonymous, 2006). In spite of being the largest producer in the world, India has to import pulses to the tune of 2 million tonnes every year to meet its domestic requirement. The major pulses in India are chickpea, pigeonpea, mungbean, urdbean, lentil and fieldpea (Ranjekar, 2003) (Table 2). Though individual crops have undergone significant changes with respect to area, production and productivity, the per capita availability of pulses has declined from 64 g/capita/day to less than 40 g/capita/day as against the recommendation of 80 g/capita/day by FAO/WHO.

Population in India is growing at 16% annually i.e., adding 16 million people every year to the already large population base of the country. More than half of all children under the age of four are malnourished and 30% of newborns are significantly underweight (Ali, 2003). It is estimated that India's population will touch nearly 1.35 billion marks by 2020 AD and will require 30.3 million tonnes double of the pulses produced presently (Ranjekar, 2003). Hence, the main challenge is to attain self-sufficiency in pulse production to meet the increasing demand of protein and ensuring the environmental security by way of checking the degradation of soil due to intensive cultivation of high input demanding crops.

Chickpea production in India

India grows chickpea on about 6.86 million hectares producing 5.35 million tonnes of grains, which represent 32 and 42% of the national pulse acreage and tonnage, respectively. Four states viz., Madhya pradesh, Rajasthan, Uttar Pradesh, and Maharashtra, together contribute 87% of the total from 65% area (Table 3). The area under chickpea has observed steady uptrend in Andhra Pradesh, Gujarat, Karnataka, Madhya Pradesh, Orissa, Maharashtra, Rajasthan and Tamil Nadu. Except Rajasthan, all other states in this category are located in southern and central zones. At present, these states contribute about 79% production from 80% of the area. As far as the productivity is concerned, chickpea has observed considerable improvement from 611 kg per ha in 1971-75 to 783 kg per ha at present in spite of being relegated to marginal and high risk prone area with shorter growth cycle due to terminal drought. The productivity of chickpea in northern states is 1.87 q/ha, and in southern and central states along with Rajasthan is 2.02 q/ ha.

Origin and domestication

Chickpea (*Cicer arietinum* L.) is thought to have originated in Anatolia (Turkey), where three closely related wild species, *C. bijugum*, *C. echinospermum* and *C. reticulatum*, are commonly found in nature (van der Maesen, 1984). Chickpea seeds had been occasionally recovered in pre-historic sites in the Near East (Renfrew, 1973). However, Ramanujam (1976) reported that remnants of chickpea radiocarbon are dated at 5450 BC and there is evidence for its cultivation in the Mediterranean basin in 3000-4000 BC. The earliest record of chickpea in northern India (Uttar Pradesh) dated at 2000 BC, and from the south India much later (Chowdhury *et al.*, 1971; Vishnu-Mittre, 1974).

Distribution of chickpea

Chickpea after dry beans and dry peas is the third most important grain legume in the world. Its cultivation is mainly confined to Asia with 90% of the global area and production. It is also grown in North and Central America, the Mediterranean region, the West Asian and North African region and Eastern Africa. However, the crop assumed greatest significance in Indian subcontinent. The crop has expanded to new niches such as Australia and Canada. Chickpea is grown mostly as a rainfed crop under conserved moisture in the post rainy season in the semi arid tropics and in

Table 1. Amino acids composition (mg/100 g protein) of protein from different sources.

Amino acid	Animal	Cereal	Pulse
Isoleucine	46.7	39.8	45.3
Leucine	79.6	86.3	78.9
Lysine	84.3	30.5	67.1
Methionine & Cystine	37.7	41.1	25.3
Tryptophan	11.4	12.1	12.3

Table 2. Protein contents in pulses.

Pulse	Protein (%)
Chickpea	22.0
Pigeonpea	22.9
Mungbean	22.1
Urdbean	21.0
Pea	19.7
Lentil	25.6

Source (Table 1-2): The Hindu Survey of Indian Agriculture, 2002.

Table 3. Area and Production of chickpea in different states of India.

State	Production (Lakh tonnes)	Area (Lakh ha)	Yield (Kg per ha)
Andhra Pradesh	96.7	142.5	679
Bihar	110.0	107.0	1028
Gujarat	41.8	81.9	512
Haryana	58.0	100.0	580
Karnataka	181.0	319.0	567
Madhya Pradesh	2493.3	2745.1	908
Maharashtra	580.0	932.0	622
Orissa	16.0	32.0	500
Punjab	6.1	6.3	968
Rajasthan	677.9	975.3	695
Tamil Nadu	5.3	8.0	663
Uttar Pradesh	779.3	822.3	948
West Bengal	30.0	27.0	1111

Source: Chickpea Research in India: Edited by Masood Ali, Shiv Kumar, N. B. Singh, IIPR, Kanpur, India.

spring and winter seasons in the temperate and Mediterranean types of climate. Kabuli types are mostly grown in the West Asian and North African region, the America and Europe, while desi types predominate in Asia, parts of Africa and Australia.

Taxonomy of chickpea

Order	Fabales
Family	Leguminosae (Fabaceae)
Subfamily	Papilionoideae (Faboideae)
Tribe	Cicereae
Genus	*Cicer*
Species	*arietinum*

Uses of chickpea

Chickpea is valued for its nutritive seeds with high protein content 12.6-30.5% (Singh *et al.*, 1997). Although chickpea is a rich source of protein, its protein quality is limited by sulphur containing amino acids, methionine and cystine. Chickpea generally meets adult human requirement for all essential amino acids except methionine and cystine (Table 4). Based on the amino acid composition, chickpea proteins were found to be of higher nutritive value as compared to other legumes (Gupta and Kapoor, 1980). The levels of different protein fractions primarily control the essential amino acid composition of chickpea seed proteins (Table 5).

Chickpea seeds are eaten fresh as green vegetables, parched, fried, roasted and boiled; as snack food, sweet and condiments; seeds are ground and the flour can be used as a soup, dhal and to make bread; prepared with pepper, salt and lemon it is served as a side dish (Saxena, 1990). Dhal is the split chickpea without its seedcoat, dried and cooked into a thick soup or ground into flour for snacks and sweetmeats (Saxena, 1990; Hulse, 1991). The protein content of Dhal is higher than that of the whole seed indicating the effect of seed coat on the protein content in chickpea genotypes (Table 6). Sprouted seeds are eaten as a vegetable or added to salads. Young plants and green pods are eaten like spinach. A small proportion of canned chickpea is also used in Turkey and Latin America, and to produce fermented food. Animal feed is another use of chickpea in many developing countries. An adhesive

Table 4. Amino acids content of chickpea.

Amino acids	g/100 g protein
Lysine	7.04
Threonine	3.52
Methionine	1.28
Cystine	1.28
Leucine	9.28
Isoleucine	5.12
Valine	4.96
Phenylalanine	5.76
Tryptophan	0.80

Table 5. Essential amino acid composition of seed protein fractions of chickpea.

Amino acid	Protein fraction (g per 100 g protein)			
	Albumin	Globulin	Glutelin	Prolamin
Lysine	10.8	6.4	6.8	2.3
Methionine + Cystine	5.3	1.8	2.6	1.5
Phe + Try	9.3	9.0	8.1	5.7
Threonine	5.4	3.5	5.7	2.2
Valine	4.5	4.2	5.7	2.1
Isoleucine	5.1	4.4	5.4	2.3
Leucine	9.8	7.5	9.1	1.6

Table 6. Nutritional composition of whole seed and dhal component of chickpea.

Constituent (%)	Whole seed	Dhal
Protein	22.0	24.5
Starch	47.3	56.0
Sugars	5.8	4.9
Ash	3.2	2.8
Fat	5.3	5.7
Crude fiber	6.3	1.1
Dietary fiber	19.0	11.3
Value (Kcal)	347.6	360.8

Source (Table 4-6): Chickpea Research in India: Edited by Masood Ali, Shiv Kumar, N. B. Singh, IIPR, Kanpur, India.

may also be prepared; although not water resistant, it is suitable for plywood. Gram husks, and green or dried stems and leaves are used for stock feed; whole seeds may be milled directly for feed. Leaves are said to yield an indigo like dye. Acid exudates from the leaves can be applied medicinally or used as vinegar. In Chile, a cooked chickpea milk (4:1) mixture was good for feeding infants, effectively controlled diarrhea. Chickpeas yield 21% starch suitable for textile sizing, giving a light finish to silk, wool and cotton cloth (Duke, 1981).

Medicinal uses

Among the food legumes, chickpea is the most hypocholesteremic agent; germinated chickpea was reported to be effective in controlling cholesterol level in rats (Geervani, 1991). "Glandular secretion of the leaves, stems and pods consists of malic and oxalic acids giving a sour taste. In India these acids used to be harvested by spreading thin muslin over the crop during the night. In the morning the soaked cloth is wrung out and the acids are collected in bottles. Medicinal applications include use for aphrodisiac, bronchitis, catarrh, cutamenia, cholera, constipation, diarrhoea, dyspepsia, flatulence, snakebite, sunstroke and warts. Acids lower the blood cholesterol levels. Seeds are considered antibilious" (Duke, 1981).

Agronomy of chickpea

Major chickpea growing areas in the country are located between 15 and 34°N latitude. It is grown throughout the country except on high altitudes of north and north eastern regions and coastal peninsula. The major chickpea producing states are M.P., Rajasthan, U.P., Maharashtra, Karnataka and Gujarat which together account for more than 85% of area and 96% of the production.

Chickpea is a quantitatively long day plant (Summerfield *et al.*, 1981) (Fig. 1). It requires cool climate for its growth and development and high temperature for maturity. The optimum temperature for its growth ranges from 15-25°C. Soil temperature of 30°C or above adversely affects the rhizobial infection in nitrogen fixation process (Dart *et al.*, 1975). The deep rooted system makes it a preferred crop in dry tracts with an annual rainfall of 60-100 cm. Thus area with moderate rainfall are considered suitable for chickpea production.

Chickpea thrives well on a wide range of soils in India including sandy (Thar Desert), Sandy loam (Entisols of north India) and black cotton soils (vertisols of

Figure 1. Different stages of chickpea growth Field view (A), root system showing nodules (B), Single young Plant (C), Pods and seeds of chickpea (D)

central India). It is highly sensitive to saline and sodic soils (Chandra, 1980). A pH range of 6-9 is favourable and an acid soil with pH of 4.6 seems to increase the problem of Fusarium wilt (Kay, 1979).

Germplasm

There are two types of chickpea i.e., desi and kabuli. Desi type has small seeds with angular shape edge and pigmented seed coat which may vary from black brown to cream or yellow whereas kabuli type has large and round seeds with white beige or pale cream seed coat. A large number of varieties suitable for different agroclimatic conditions have been recommended for cultivation in India (Table 7).

Fertilizer Management

Judicious and balanced use of nutrient is required not only for quantitative and qualitative increase in yield but also to help plant growth vigorously and overcome biotic and abiotic stresses. Total uptake of nutrients by chickpea crop has been estimated at 60-200 kg N, 5-15 kg P and 60-170 kg K per ha (Ahlawat, 2000). In general, chickpea has shown response to application of nitrogen upto 15-20 kg/ha. However, response to nitrogen is poor under efficient biological nitrogen fixation system when nodules develop properly (Saxena and Sheldrake, 1980). Field experiments conducted in the Indo-gangetic plains of the country suggest 19-63% yield increase in chickpea due to inoculation with rhizobium (Ali *et al.*, 2002). When chickpea is to be cultivated after potato, maize, sorghum, or late duration rice, a greater nitrogen dose (25-50%) is recommended due to low soil nitrogen (Ali and Mishra, 2000). Babu *et al.* (2000) have, however, reported that only 50% of the recommended dose of nitrogen was sufficient to realize higher yield of chickpea grown after potato. Under late sown condition, the crop responds well upto 40 kg/ha. Foliar application of 2% urea at the time of pod formation is useful (Bharud, 2001; Dudhade and Patil, 2001; Anonymous, 2003).

Phosphorus is the most important nutrient for chickpea and increases protein content in the grains (Guhey *et al.*, 2000). Conspicuous response of phosphorus application in chickpea has been observed by several workers (Dev, 1987; Prabhakar and Saraf, 1991). Among various sources of phosphorus, single super phosphate (SSP) has been found more effective over DAP (Anonymous, 1994a). Phosphate solubilizing bacteria (PSB) have been found effective in increasing the efficiency

Table 7. Cultivars of chickpea recommended for cultivation in different states in India.

States	Recommended Cultivars
Andhra Pradesh	ICCV-2, ICCV-37, ICCV-4, ICCV-10
Assam	KWR-108, BG-256, L-550, KPG-59
Bihar	KWR-108, BG-256, Avrodhi, Pant G-114, Pusa-209, L-550, Pusa-1003
Gujarat	Pusa 391, Vijay, ICCV-10, ICCV-4, Pusa-240, GG-1, Pusa-1053
Haryana	Haryana Chana-1, GNG-469, Pusa-362, Gora Hisari, Karnal Chana, Gaurav, H-208, H-335, Pusa-1053
Himachal Pradesh	BBG-1, Haryana Chana-1, L-550
J&K	GNG-469, L-550, PBG-1, Haryana Chana-1
Karnataka	BDN 9-3, ICCV-10, ICCV-2, Annegiri-1
Madhya Pradesh	JG-74, JG-315, Vijay, Pusa-256, Phule G-5, Pusa-1053
Maharashtra	Vijay, Phule G-5, Vishal, ICCV-10, Pusa-1053
Orissa	Radhey, ICCV-10, L-550, Pusa-372, Pusa-1003.
Punjab	PBG-1, GNG-469, Haryana Chana-1, Gaurav, L-550, C-235, G-543, Pusa-1053, GPF-2, PDG-3
Rajasthan	GNG-416, GNG-469, GNG-663, PBG-1, L-550, Pusa-256, RSG-44, Pusa-1053, PDG 84-1
Tamil Nadu	ICCV-10, BDN 9-3, CO-3, CO-4
Uttar Pradesh	KWR-108, Avrodhi, BG-256, K-850, Pant G-186, Pusa-372, Radhey, JG-315, Uday (KPG-75), Pusa-1003, Pusa-1053
West Bengal	Pusa-372, KWR-108, KPG-59, BG-256, Pusa-1003
North Eastern States	KWR-108, Avrodhi, KPG-59, BG-256

of applied phosphorus. Application of PSB culture either with DAP or SSP at 40 kg P_2O_5/ha in calcareous soil, where phosphorus fixation is a common phenomenon was found to improve chickpea yield and available phosphorus status of the soil (Saad and Sharma, 2001). Foliar application of 2% DAP has been found beneficial in increasing the seed yield of chickpea under rainfed conditions in North East Plain Zone, North West Plain Zone, Central Zone and Southern Zone of the country.

Potassium

The response of chickpea to potassium (K) is small and of seldom significance. The response to applied K has been reported in laterite, red, coastal and deltic alluvial soil of India. Generally application of 20 kg K_2O/ha is recommended under deficient soil conditions. Indian soils are rich in available potassium hence rarely found a place in fertilizer schedule of chickpea (Subbarao and Srinivasarao, 1996).

Sulphur

Sulphur, being a constituent of predominant amino acids (methionine, cysteine and cystine), ferrodoxin containing nitrogenase and also involved in metabolic activities of vitamins, thiamine and coenzyme and is greatly highly required by chickpea crop. Study conducted by Srinivasarao *et al.* (2002) in different pulse growing regions revealed low to medium range of available sulphur in Indian soil. In sulphur deficient soil, the response to applied sulphur has been found in the range of 20-60 kg S/ha (Aulakh and Pasricha, 1986). Ali and Singh (1995), however, have recorded significant increase in plant growth and yield of chickpea response upto 40 kg S/ha (Ali and Singh, 1995).

Trace elements

Among the micronutrients, zinc deficiency is widely observed followed by boron and iron. Zinc is also found to reduce the growth of collar rot, wilt and root rot in chickpea (Gupta, 1999). Yield gain of 320 kg/ha with the application of 25 kg $ZnSO_4$/ ha has been observed (Takkar and Nayyar, 1986). Boron deficiency is an emerging problem in some states like Orissa (69% samples deficient), Bihar (39%), U.P. (23%) and Gujarat (5%) (Takkar, 1996). Soil application of 10 kg borax/ha has given yield advantage to the tune of 306-405 kg/ha in chickpea (Ali and Mishra, 1996). Seed treatment of chickpea with Molybdenum and Iron can synergise the effect of

applied phosphorus, PSB and rhizobium in terms of yield and nodulation (Sarawgi *et al.*, 1999).

Diseases of chickpea

Diseases are the most serious constraints in chickpea production causing upto 100% losses (Table 8). Environmental factors and intensity of abiotic stresses may influence the incidence of the diseases, e.g. yield losses due to wilt and root rot diseases increase under drought and high temperature situation in the country (Gurha *et al.*, 2003). Due to nutritive nature of plants, a large number of plant pathogens have been found associated with chickpea. A careful examination relevant informations indicate that 67 fungi, 3 bacteria, 22 viruses and 80 nematodes may infect the crop and cause a disease (Nene and Reddy, 1987; Haware, 1998; Singh and Sharma, 1998; Singh *et al.*, 1999). Among these Fusarium wilt, root rots, Ascochyta blight and Botrytis grey mold are major diseases and may cause significant yield losses to chickpea plants (Gurha *et al.*, 2003). In addition to fungal diseases, root-knot nematode diseases caused by *Meloidogyne incognita* and *M. javanica* are widely distributed nematodes in India (Khan, 1997) and may cause 19-40% economic loss (Ali *et al.*, 2003). Ali (1997) reported 22-84% avoidable loss due to *M. javanica* and 25-60% due to *M. incognita*. Another species of root-knot nematodes *M. arenaria* caused 39% loss in grain yield of chickpea in Gujarat (Patel, 1997). Mhase *et al.* (1997) estimated 42% yield loss due to root-knot nematode in Maharashtra. Information gathered from different sources on avoidable yield loss due to root-knot nematodes in chickpea indicated 17-60% losses in Bihar, 17-56% in Gujarat, 8% in Haryana, 35-43% in Maharashtra, 22% in Punjab, 17-60% in Rajasthan and 22-23% in Uttar Pradesh (Ali *et al.*, 2003). In addition to direct damage, root-knot nematodes synergise other pathogens to cause greater damage (Khan, 1993). The nematode infection is known to break resistance in many wilt resistant cultivars of chickpea viz., Avrodhi, ICC 12275, ICC 11322, ICC 11319 and ICC 12272. Nematode infestation changes the physiology of the host plants, thus making the task easier for fungus to attack and cause the disease. In susceptible genotypes, nematode advance the onset of wilt from 31-16 days and increases the disease incidence from 25-50% (Ali *et al.*, 2003). The presence of both organisms together in soil result in severe and sudden loss to the crop after emergence (Ramnath and Dwivedi, 1981).

Table 8. Disease of chickpea caused by various pathogens.

Disease	Causal organism
Fungal diseases	
Acrophialophora wilt	*Acrophialophora fusispora*
Alternaria blight	*Alternaria alternata Alternaria tenuissima*
Aphanomyces root rot	*Aphanomyces euteiches*
Ascochyta blight	*Ascochyta rabiei* (races 1, 2, 3, 4, 5, and 6) *Mycosphaerella rabiei* = *Didymella rabiei* [teleomorph]
Black root rot	*Fusarium solani*
Black streak root rot	*Thielaviopsis basicola*
Botrytis gray mold	*Botrytis cinerea*
Collar rot	*Sclerotium rolfsii Athelia rolfsii* = *Corticium rolfsii* [teleomorph]
Colletotrichum blight	*Colletotrichum capsici Colletotrichum dematium*
Cylindrocladium root rot	*Cylindrocladium clavatum*
Damping-off	*Pythium debaryanum Pythium irregulare Pythium ultimum*
Downy mildew	*Peronospora* sp.
Dry root rot	*Macrophomina phaseolina* = *Rhizoctonia bataticola*
Foot rot	*Phacidiopycnis padwickii* = *Operculella padwickii*
Fusarium root rot	*Fusarium acuminatum Fusarium arthrosporioides Fusarium avenaceum Fusarium equiseti Fusarium solani f. sp. eumartii* = *Fusarium eumartii*
Fusarium wilt	***Fusarium oxysporum* f. sp. *ciceri* (races 0, 1, 2, 3, 4, 5, and 6)**
Myrothecium leaf spot	*Myrothecium roridum*
Mystrosporium leaf spot	*Mystrosporium* sp.
Neocosmospora root rot	*Neocosmospora vasinfecta*
Ozonium collar rot	*Ozonium texanum* var. *parasiticum*
Phoma blight	*Phoma medicaginis*
Phytophthora root rot	*Phytophthora citrophthora Phytophthora cryptogea Phytophthora drechsleri Phytophthora megasperma*
Pleospora leaf spot	*Pleospora herbarum Stemphylium herbarum* [anamorph]
Powdery mildew	*Leveillula taurica Oidiopsis taurica* [anamorph] *Erysiphe* sp.

Continued-----

Continued----- Table 8

Seedling or seed rot	*Aspergillus flavus Trichothecium roseum*
Stemphylium blight	*Stemphylium sarciniforme*
Trichoderma foot rot	*Trichoderma harzianum*
Verticillium wilt	*Verticillium albo-atrum Verticillium dahliae*
Wet root rot	*Rhizoctonia solani*

Bacterial diseases

Bacterial blight	*Xanthomonas campestris* pv. *cassiae*
Bacterial leaf spot	*Pseudomonas andropogonis*

Nematodes

Dirty root (reniform nematode)	*Rotylenchulus reniformis*
Pearly root (cyst nematode)	*Heterodera ciceri Heterodera rosii*
Root-knot (root-knot nematode)	*Meloidogyne arenaria Meloidogyne artiellia Meloidogyne incognita Meloidogyne javanica*
Root lesion (root lesion nematode)	*Pratylenchus brachyurus Pratylenchus thornei*

Viral diseases

Bushy stunt	Chickpea bushy stunt virus
Distortion mosaic	Chickpea distortion mosaic virus
Filiform	Chickpea filiform virus
Mosaic	Alfalfa mosaic virus
Narrow leaf	Bean yellow mosaic virus
Necrosis	Lettuce necrotic yellows virus Pea streak virus
Proliferation	Cucumber mosaic virus
Stunt Bean (pea)	Leaf roll virus
Yellowing	Pea enation mosaic virus

Phytoplasmal diseases

Phyllody	Mycoplasma-like organism

Reduction in growth of chickpea was observed when both *M. javanica* and *F. oxysporum* f. sp. *ciceri* were present together in the soil (Ali and Gurha, 1994). The damage was greater when nematode was inoculated 30 days prior to the fungus (Goel and Gupta, 1986). The wilting percentage becomes higher when both the organisms are present. Maximum wilting in plants was observed when the *F. oxysporum* f. sp. *ciceri* and *M. incognita* were inoculated simultaneously (Patel *et al.*, 2000).

Chickpea wilt (*Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. f. sp. *ciceri* (Padwick) Snyd. & Hans.)

Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceri* was first reported from India by (Butler, 1918). It is one of the most serious diseases in most of the chickpea growing countries of the world. Annual chickpea yield losses from Fusarium wilt vary from 10-15% (Jalali and Chand, 1992; Trapero-Casas and Jimenez- Diaz, 1985), but the disease can destroy the crop completely under specific conditions. Srivastava *et al.* (1984) observed the yield losses ranging from 10-90%. Nema and Khare (1973) observed damage upto 61% at seedling stage and 43% at flowering stage. Early wilting reduced the seed number/plant and caused more yield losses than late wilting (Haware and Nene, 1980). Sattar *et al.* (1953) have estimated an annual loss of 12 million rupees in Pakistan. In India, the disease is estimated to cause 10% yield loss but figure on monetary losses is not available (Singh and Dahiya, 1973).

Taxonomic position of *Fusarium oxysporum* f. sp. *ciceri*

Division	Ascomycota
Sub division	Pezizomycotina
Class	Sordariomycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*
species	*oxysporum*
Forma specialis	*ciceri*

Distribution

The chickpea wilt is economically very important and has been reported from several countries including India, Bangladesh, Burma, Ethopia, Mexico, Pakistan, Syria and Tunisia (Nene *et al.*, 1984) Chile, Iran, Sudan, United States (Haware *et al.*, 1986), Peru (Echandi, 1970), USSR (Stepanova, 1971) and Malawi (Kannaiyan, 1981) (Fig. 2). However, it is more severe in India, Iran, Pakistan, Nepal, Burma, Spain and Tunisia. Disease occurs in almost all types of chickpeas. In general, spring sown crop is more vulnerable to wilt than winter sown (Hawtin and Singh, 1984). Because of the extreme temperature shift in the northern belt of India early wilting occurs around November, and late wilting at flowering stage around February-March. In winter during December-February the wilt incidence remains at the lowest.

Symptoms

Generally the disease occurs at two stages of plant growth i.e., seedling stage, flowering stage or adult stage. The chief symptoms of the disease are: yellowing and drying of leaves from base upward, drooping of petioles and rachis, improper branching, withering of plants, browning of vascular bundles, and finally wilting of plants (Prasad and Padwick, 1939; Argikar, 1970; Westerlund *et al.*, 1974). Chauhan (1962a) reported the initial symptom of the disease to be acropetal vein clearing of leaves. When the disease occurs at seedling stage (3-5 weeks after sowing), the seedlings collapse and lie flat on the soil surface, although they retain normal green colour. Such diseased seedlings, when uprooted, generally show uneven shrinking of the stem above and below the collar region. The affected seedlings do not show any external root rot; however, when split open vertically from collar region downward, black discoloration of xylem vessels is visible. The seedlings of highly susceptible cultivars which die within 10 days after emergence may not exhibit black discoloration of internal tissues; nevertheless, internal browning from root tip upward will be visible.

In the case of infection of adult plants (6-8 weeks after sowing) are infected, the diseased plants exhibit drooping of petioles, rachis, and leaflets. Initially, drooping is observed in the upper part of the plants, but within a short time (1-2 days) it is visible on the entire plant. The lower leaves dry but do not shed at maturity. Murumkar and Chavan (1985) noted physiological changes taking place in leaves infected by the fungal pathogen (reduction in chlorophyll and increase in organic

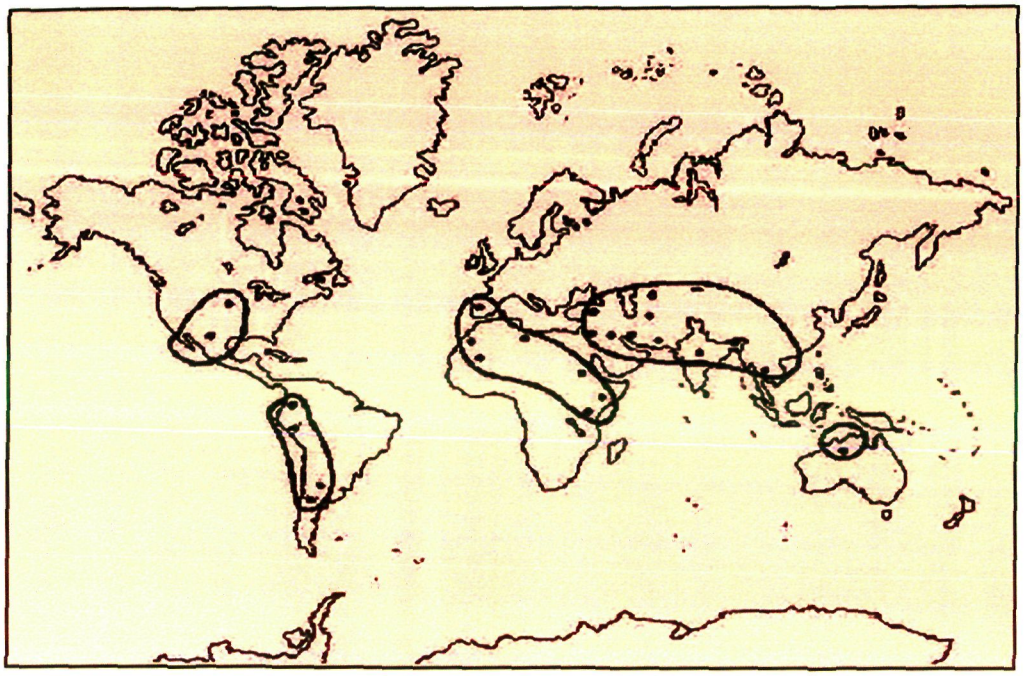


Figure 2. World distribution map of the wilt disease of chickpea

acids, polyphenols and carbohydrates). In a similar study the number of chloroplasts and starch formation in the mesophyll cells decreased following infection by the pathogen (Chauhan, 1962a).

The causal organism

The fungus is septate, profusely branched; growing on potato sucrose/dextrose agar at 25°C initially white turning light buff or deep brown later, fluffy or submerged. The growth becomes felted or wrinkled in old cultures. Various types of pigmentations (yellow, brown, crimson) may be observed in culture. On solid medium, microconidia may be usually borne on simple and short conidiophores which arise laterally on the hyphae. They are oval to cylindrical, straight or curved and measure 2.5-3.5 x 5-11 mm. Macroconidia are borne on branched conidiophores, thin walled, 3 to 5 septate, fusoid, pointed at both ends and measure 3.5-4.5 x 25-65 mm. Chlamydospores are formed in old cultures, which are smooth or rough walled, terminal and intercalary and may be forked singly or in pairs or in chains (Gupta *et al.*, 1986).

Two pathotypes have been distinguished within *F. oxysporum* f. sp. *ciceri* based on the distinct yellowing or wilting syndromes they cause in chickpeas (Chauhan, 1962b). The yellowing pathotype induces progressive foliar yellowing with vascular discoloration, while the wilting pathotype induces severe and fast chlorosis, flaccidity and vascular discoloration.

Epidemiology

The fungus may be seedborne and may survive in plant debris in soil (Westerlund *et al.*, 1974; Nene *et al.*, 1980; Kumar *et al.*, 1983; Sharma and Gupta, 1986; Padwick, 1941). Kumar *et al.* (1983) isolated the pathogen from seed samples obtained from five sources. Haware *et al.* (1978) showed the fungus to be in the hilum of the seed in the form of chlamydospore like structures. The pathogen survives well in roots and stems, even in apparently healthy looking plants growing among diseased chickpea harboring enough fungus (Padwick, 1941). Haware and Nene (1982) found pigeonpea, lentil and pea as symptomless carriers of the disease. The pathogen may also parasitize several weeds such as *Cyperus rotendus*, *Tribulus terrestris*, *Convolvulus arvensis* and *Cardiospermum halicacabum* (Nene *et al.*, 1980).

The soil type, reaction, moisture and temperature are known to influence disease development. The greenhouse studies substantiate the fact that disease is

more severe in light sandy soils than heavy clay ones (Echandi, 1970; Kotasthane *et al.*, 1979). Chandra *et al.* (1974) attributed higher disease severity in light sandy soils to low water retention ability of these soils. Soil pH may also influence the disease intensity that increased with lowering pH, being considerably low at pH 9.2 (Chauhan, 1962c). However, Shaikh (1974) reported that the pathogen tolerated a wide range of pH, with an optimum of pH 5.0-6.5. High soil temperature and deficiency of moisture appear to have a definite bearing on the incidence of the disease (Baker and Cook, 1974; Chauhan, 1963). Lower levels of soil moisture (10%) kept the plant mortality low due to the disease, though 12% of the plants were damaged, as compared to 83% in soil with moisture at 25% level (Sinha, 1973). Soil temperature relations showed that the disease is optimum at 25°C and is at a lower ebb at 20°C. The amount of organic matter (Chauhan, 1965) and humus (Chauhan, 1962d) content of the soil were found inversely related to wilt incidence.

Root-knot nematode disease of chickpea

Phytonematodes are another important group of pathogens and cause considerable damage to pulse crops (Sasser, 1989). Ali (1995) listed 97 nematode species associated with chickpea on global basis, out of which 64 have been reported from India, but the major damage is caused by three endoparasitic nematodes viz., *Meloidogyne* spp., *Heterodera* spp. and *Rotylenchulus reniformis*, which are known to inhabit inside the roots (Ali *et al.*, 2003). The former happens to be the major nematode in all types of chickpea. Three species of the root-knot nematode, *M. incognita*, *M. javanica* and *M. arenaria* are associated with chickpea. Of these species, *M. incognita* is apparently the most predominant which is closely followed by *M. javanica* (Sharma and Sharma, 1998). The nematode is polyphagous in nature, and possesses a threat to pulse cultivation in India (Bhagwati and Phukan, 1991). The pathogenicity of *Meloidogyne* spp. has been proved on chickpea, pigeonpea, cowpea, mungbean, pea, lentil, urdbean, rajmash and several other pulses (Gupta *et al.*, 1986, Khan *et al.*, 2004).

Distribution and economic importance

Root-knot nematodes are widely distributed throughout the world, especially in tropical, subtropical and mediterranean climates (Sasser, 1979). Out of the described species of *Meloidogyne, M. incognita* with 4 races (R_1, R_2, R_3, and R_4), *M. javanica,*

M. arenaria with two races ($R_1$and R_2) and *M. hapla* have wide host range and distribution (Sasser, 1979, Khan *et al.*, 1988). In India 10 species viz., *M. incognita, M. javanica, M. arenaria, M. hapla, M. graminicola, M. exigua, M. africana, M. bravicaudata, M. graminis* and *M. triticoryzae* are present. The four species i.e., *M. incognita, M. javanica, M. arenaria* and *M. hapla* have been observed from all the states and Union Territories of India. Occurrence of races of *M. incognita* and *M. arenaria* has also been confirmed in India. All the four races of *M. incognita* (R_1, R_2, R_3, and R_4) and one race of *M. arenaria* (R_2) are present in U.P. In other states one or two races of *M. incognita* have been recorded. Root-knot nematodes (*M. incognita* and *M. javanica*) have been reported to cause 19-40% economic loss to chickpea in India (Fig. 3) (Ali *et al.*, 2003).

Disease symptoms

The nematode causes nonspecific above ground symptoms which resemble to mineral deficiency. These symptoms appear in the patches of plants in a field and are characterized by stunted growth, yellowing and/or chlorosis of foliage. The leaves may dehiscent prematurely. Beaded appearance of roots and root-knot galls on root system is the most characteristic symptom of root-knot nematode infection (Ali *et al.*, 2003). Poor emergence and death of young seedlings may occur in heavily infested soil, but death of grown-up plants is rare unless some fungus or bacteria become associated and form a disease complex (Francl and Wheeler, 1993). *Meloidogyne incognita* also causes significant reduction in nodulation, nitrogenous activity of nodules and nitrate reductase activity of leaves in chickpea (Chahal and Chahal, 1991). The rhizobium, however, does not interfere with the nematode pathogenesis (Taha, 1993).

Causal organism

Root-knot nematode, *Meloidogyne* spp. is a sedentary endoparasite and second stage juveniles are infective stage of the nematode. Male and female larvae penetrate host roots. The females become sedentary after getting suitable site for feeding and gradually assume obesity. Male larvae do not feed and remain or become vermiform and migrate out of root at maturity. Females at maturity lay hundreds of eggs, on average 200-500 in a gelatinous matrix collectively called as an egg mass. Reproduction is parthenogenetic and life cycle from egg to egg completes in around

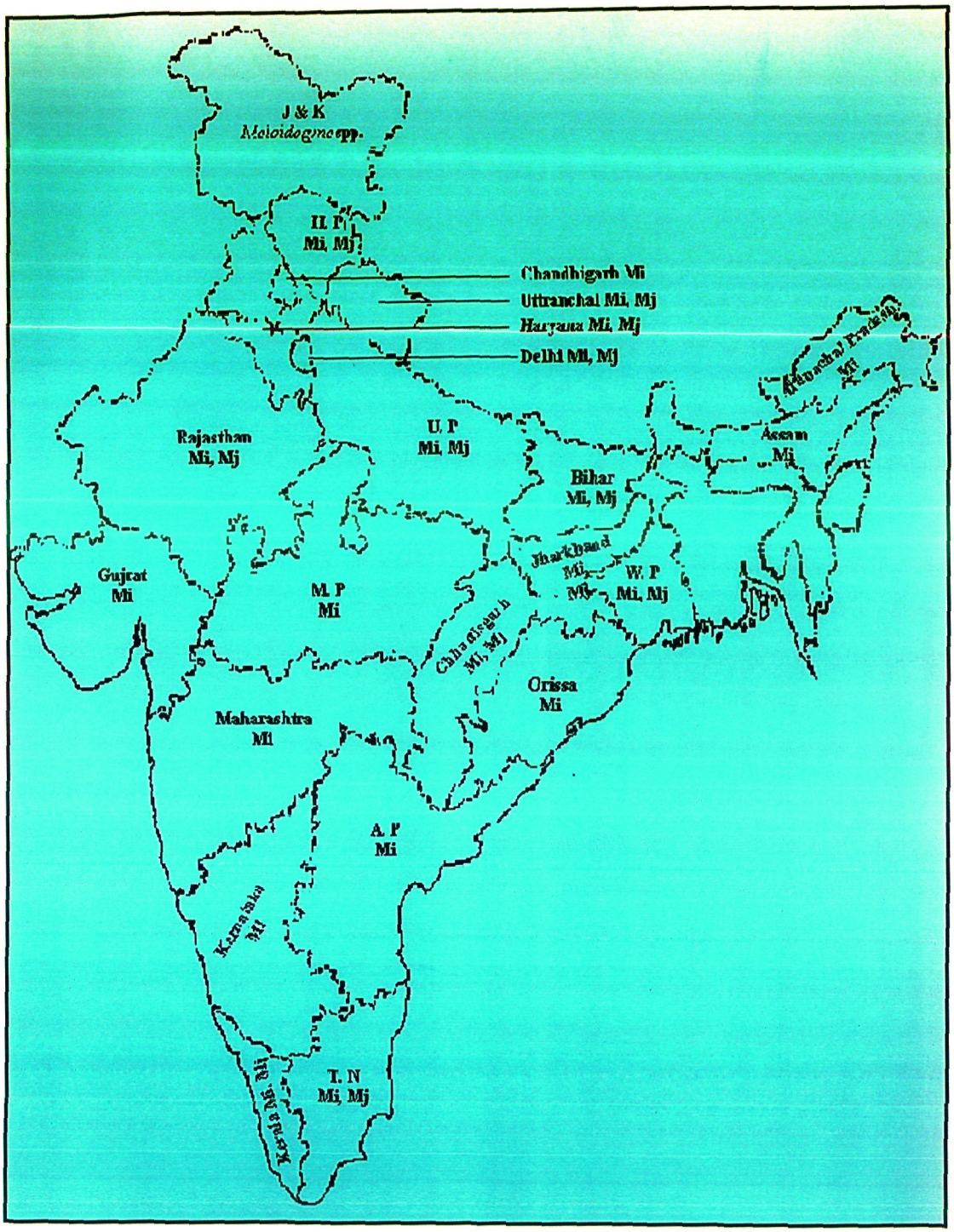


Figure 3. Distribution of *Meloidogyne incognita* (Mi) and *M. javanica* (Mj) in different states of India.

three weeks. The species of root-knot nematode *M. incognita* and *M. javanica* which have been found associated with root-knot of pigeonpea can be differentiated on the morphology of perineal pattern, female stylets, male heads and stylets, and second stage juveniles (Eisenback, 1985).

Life cycle and disease development

The life cycle starts from eggs. After embryonic development, larva is formed inside the egg. The first molt occurs inside the egg. Under suitable environmental conditions (temperature, moisture and osmotic pressure) an egg hatches giving rise to second stage juveniles. The J_2 is infective stage of the nematode. The larvae moves slowly and randomly in the soil and does not feed. The larvae, however, moves faster and in specific direction when it has received chemical stimuli through root exudates from a susceptible plant. The second stage juveniles penetrate the roots and move mostly between the undifferentiated root cells till their head reach in endodermis. After locating a suitable site for feeding in primary phloem the J_2 induces formation of giant cells (2-6) around the head region (Haung, 1985; Pasha *et al.*, 1987). After formation of giant cells, the juveniles come to rest (sedentary) with the head in the phloem and the body in cortex and feeds strictly on giant cells around the head region. The giant cells are formed due to repeated endomitosis without cytokinesis of primary phloem cells or adjacent parenchyma and pericycle cells (Hussey, 1989; Wiggers *et al.*, 1990; Haung, 1985; Pasha *et al.*, 1987). The juveniles feed on giant cells for several days, as a result nematode body width increases and structural changes in the body also occur. Second and third molts occur and the female becomes pyriform. The sexes can be differentiated at all stages, as the males have one gonad where females have a pair. The males at maturity migrate out of the root, wander in soil and soon die. The fourth stage female continues to grow in size and undergoes the fourth molt to become the adult female. Reproduction is parthenogenetic and fertilized eggs develop without mating. The egg laying is completed in a week. The hatched juveniles from the new eggs may infect the same root tissue to establish new infections and form secondary galls. The hatched larvae may also infect other adjacent plants whose roots have come near the site of primary infection. The length of life cycle and number of generations depends on host health and temperature. Optimum temperature for *M. incognita* and *M. javanica* is 25-30°C. The life cycle of these species completes in 21-25 days at 26-27°C and in 50-60 days or even 80

days at 14-16°C. In general, temperatures of 25-28°C and light textured soil are best for rapid multiplication, larval movement, infection and gall formation.

The second stage juveniles (infective stage) inject secretions from oesophageal glands, which lead to several physiological and morphological changes in the invaded tissue, paramount significance are giant cells (Bird, 1972). Concurrent with the establishment of giant cells, root tissue around the nematode and its feeding site undergoes hyperplasia and hypertrophy leading to development of characteristic root galls which develop within two days of penetration and are often the first discernible symptoms of nematode infection (Khan and Esfahani, 1992). Formation of galls causes impairment of absorption of water and minerals by roots, subsequently the plant show water stress symptoms (Wallace, 1987). Upward conduction also gets impaired leading to accumulation of nutrients in root tissue (N, P, K, Mg etc.).

Fungus-nematode wilt disease complex

The intensity of disease caused by the nematodes often gets aggravated in presence of other microorganisms like fungi, bacteria, virus, mycoplasma etc. Among the most common fungi found to be associated largely with root-knot nematode, are the species of *Fusarium* followed by *Rhizoctonia solani*. The interaction with wilt pathogens leads to: i). increase in severity of the disease, ii). breakdown of resistance to wilt pathogens, iii). predispose plants to fungi. Root-knot nematodes are known to break resistance in many wilt resistant cultivars of chickpea viz., Avrodhi, ICC 12275, ICC 11322, ICC 11319 and ICC 12272 (Ali *et al.*, 2003). Nematode infestation changes the physiology of the host plants, thus making the task easier for fungus to attack and cause the disease. In susceptible genotypes, nematodes advance the onset of wilt from 31 to 16 days and increase the disease incidence from 25-50%. The presence of both organisms together in soil result in severe and sudden loss to the crop after emergence (Ramnath and Dwivedi, 1981). Reduction in various growth parameters was observed when nematode was inoculated 30 days prior to the fungus (Goel and Gupta, 1986). The wilting percentage becomes higher when both the organisms are present. Histopathologically, roots inoculated with nematode or in combination with fungus show typical giant cell formation, development of egg masses, larvae and female nematode inside the roots. Hypertrophic areas in vascular parenchyma as well as in the parenchyma of cortex cells around

nematode body are either collapsed or compressed due to presence applied by tissue development of nematode. These roots are invaded and colonized by the wilt fungus. This makes the host susceptible to wilt damage by breaking its resistance (Upadhyay and Dwivedi, 1987). Roots of such wilted plants show no external rotting, drying or discoloration. Seedlings show rotting at the collar region and downwards. Affected seedlings usually occur in small scattered patches in the field.

The interaction of *Fusarium oxysporum* f. sp. *ciceri* with *M. incognita* on chickpea cv. Dahod Yellow revealed that the organisms, either individually or in combination, significantly reduced plant height and fresh root and shoot weights. The reduction caused by *M. incognita* was greater compared to *Fusarium oxysporum* f. sp. *ciceri*. Among combined inoculations, the simultaneous inoculation of both organisms had maximum suppressive effect on the growth of chickpea plants compared to the preceding or succeeding inoculation of *F. oxysporum* f. sp. *ciceri* and *M. incognita*. Root galling and *M. incognita* multiplication on chickpea, which were maximum when *M. incognita* was inoculated alone, were reduced in the presence of *F. oxysporum* f. sp. *ciceri*. The fungus alone was able to produce wilt disease but the incubation period for disease development was reduced and the severity of the disease increased when *M. incognita* was present with the fungus. Maximum wilting of plant was observed when the *F. oxysporum* f. sp. *ciceri* and *M. incognita* were inoculated simultaneously (Patel *et al.*, 2000).

The disease intensity of black root rot caused by *F. solani* in chickpea increased when root-knot nematode was inoculated with it. This combination also shortened the incubation period for disease expression (Mani and Sethi, 1987). Significant reduction in fresh shoot weight and number of nodules per plants has also been observed (Dalal and Bhatti, 1985). Synergistic interaction between fungus and nematode in chickpea, pigeonpea, lentil, mungbean, fieldpea and common bean have been observed by different researchers where the presence of nematodes increased the severity of disease. The disease intensity of wet root rot of chickpea increased in combination with root-knot nematodes. The root rot index was high when inoculation of the nematode precedes that of *F. solani* (Mani and Sethi, 1987).

Concomitant inoculation of *M. javanica* and *Rhizoctonia bataticola* in chickpea seedlings reduce plant growth. The top roots were devoid of the lateral and finer roots and became dark, showing sign of rotting (Ali *et al.*, 2003). The disease intensity of collar rot caused by *Sclerotium rolfsii* was highest when the plants were

infested simultaneously with fungus and root-knot nematode (Ali *et al.*, 2003). Inoculation of *M. javanica* juveniles prior to *F. oxysporum* f. sp. *ciceri* caused greater wilt incidence in susceptible cultivars and induced vascular discoloration in roots of resistant cultivars of chickpea (Maheshwari *et al.*, 1997). *Meloidogyne* spp. increased *Fusarium* wilt on tomato (Goode and McGuire, 1967), brinjal (Noguera and Smits, 1982), squash (Caperton *et al.*, 1986), chickpea (Maheshwari *et al.*, 1997), onion (Alice *et al.*, 1996) and rhizome rot of ginger (Makhnotra *et al.*, 1997).

Root-knot nematodes are not only responsible for increasing the incidence and the severity of wilt diseases, but they also break resistance to Fusarium wilt in different crops. Jenkins and Coursen (1957) reported that *M. incognita* promoted 100% wilt symptoms in *Fusarium* resistant cv. Chesapeake while *M. hapla* could produce only 66% wilt symptoms. *Meloidogyne incognita* along with *R. solani* reduced the germination of brinjal and tomato seeds, causing severe damping-off compared to the fungus alone (Arya and Sakena, 1988; Chahal and Chabra, 1984). Synergistic interactions between *Meloidogyne* species and *S. rolfsii* have been observed on tobacco, brinjal and groundnut (Reddy *et al.*, 1972; Starr *et al.*, 1996).

Mechanism of fungus-nematode wilt disease complex

The overall mechanism of interactions between wilt fungi and root-knot nematodes is not fully understood (Mai and Abawi, 1987). Sequential or concomitant infection by root-knot nematodes and *Fusarium* spp. may increase the wilt severity and death rate of plants. The initial phase of the interaction occurs in rhizosphere, where root exudates from root-knot infected plants stimulate the fungal pathogen (Mac Hardy and Beckman, 1981). The exudates also suppress actinomycetes that antagonize the wilt fungus (Cook and Baker, 1983).

The next phase in these interactions involves the effect of root-knot infection on invasion by the wilt fungus. Initially it was thought that micropunctures caused by nematodes on plant root facilitate entry of the fungal plant pathogens (Huisman and Gerik, 1989). Later it was demonstrated that severity of wilt diseases increased greatly when root-knot nematodes were added three to four weeks prior to fungus inoculation of the host in comparison to simultaneous inoculations of both the pathogens. This led to the realization that the mechanism involved in the interaction are physiological rather than physical (Khan, 1993).

The ultimate phase of interaction between two pathogens occurs during the pathogenesis of the wilt fungus is the modifications in the host plant by root-knot nematodes which can be considered a key leading to increased wilt severity of the disease complex. The sedentary females of *Meloidogyne* spp. establish their feeding sites in the phloem parenchyma cells, bringing about significant changes in the morphology, anatomy and biochemistry of the host plant. Thus it is probably the major site of interaction between these pathogens (Mai and Abawi, 1987). Feeding sites induced by *Meloidogyne* spp. are called as giant cells which are in a state of high metabolic activity through the continous stimulation by the nematode (Webster, 1975). The giant cells contain greater amount of DNA, RNA and photosynthates especially three to four weeks after infection (Bird, 1972; Wiggers *et al.*, 1990). Concentration of sugars, hemicellulose, organic acids, free amino acids, proteins and lipids is also greater during the late stage of infection (Haung, 1985). This enriched medium benefits the fungal pathogens. The giant cells remain in a perpetual state which delays maturation and suberization of other vascular tissues, and thus *Fusarium* successfully penetrates and establishes in the xylem elements. Inhibition of tylosis formation by root-knot nematodes is also offered as a possible mechanism for increased wilt severity. Tylosis formed in the xylem vessels by expansion of xylem parenchyma through the pits do not develop from xylem parenchyma cells which are transformed into giant cells or physiologically altered adjacent cells (Webster, 1985).

Thus it may be concluded that a synergistic relationship is the common feature of fungus-nematode interaction, and fungus diseases may become more pronounced and appear earlier when plants are infected with nematodes (Evans, 1987; Mai and Abawi, 1987; Francl *et al.*, 1988; Hasan, 1988; 1989; Gray *et al.*, 1990). Some soil fungi which are normally known to be non-pathogenic on plants become pathogenic on roots infected with nematodes (Powell, 1971). In general, in synergistic interaction nematodes provide an opportunity to fungal pathogens to show greater aggressiveness and to cause greater pathogenic damage to the host.

The severity of wilt disease complex of chickpea was increased when 100 or 1000 *M. incognita* J_2/500 g soil was inoculated along with 1 and 2 g of *Fusarium oxysporum* f. sp. *ciceri*. The incubation period for disease expression was also shortened (Mani and Sethi, 1987). Dwivedi *et al.* (1992) reported that growth of pigeonpea plants inoculated with *M. incognita* and *F. udum* simultaneously was

suppressed greater than those inoculated with *F. udum* alone. Root colonization by *F. oxysporum* in mungbean was significantly greater in the presence of *M. incognita* (Shahzad and Ghaffar, 1996). Khan and Salam (1996) observed synergistic interaction between *M. incognita* and *F. udum* on pigeonpea. They found that wilting appeared earlier and more severe in concomitantly inoculated plants than those inoculated with the fungus alone. Marley and Hillocks (1996) reported that wilt resistant cultivars of pigeonpea became susceptible to *F. udum* in the presence of *M. incognita* and *M. javanica.* Singh and Goswami (2001) reported that wilting of cowpea increased when plants were inoculated with *M. incognita* and *F. oxysporum* as compared with *F. oxysporum* alone.

Reciprocal effect of the wilt fungus on nematode pathogenesis is negative. The wilt fungus, in general, dominates in the interactions, and suppresses the development and reproduction of the participating nematode. Some pathogenic fungi produce metabolites that suppress hatching of nematode juveniles (Vaishnav *et al.*, 1985; Mani *et al.*, 1986; Ciancio *et al.*, 1988). The population density of nematodes in the interaction is adversely affected by the activity of the fungal pathogen. Soil population of sedentary endoparasites such as *Meloidogyne* and *Heterodera* show decreased population density in the presence of wilt fungi (*Fusarium* or *Verticillium*) (Salem, 1980; Hasan, 1984; Nordmeyer and Sikora, 1983; Al-Hazmi, 1985; Griffin and Thyr, 1986; Starr and Veech, 1986; Griffin *et al.*, 1988; Hasan, 1989; Starr *et al.*, 1989; Gray *et al.*, 1990; Fazal *et al.*, 1994). The repression of sedentary endoparasites has been implicated with the impairment of nutrient supply through giant cells or syncytia to the developing and egglaying female nematodes. The food shortage causes starvation to females or their sex reversal (Triantaphyllou, 1960). *Fusarium* spp. actively colonized gaint cells and consume their contents as a result the female nematode is deprived of feeding and consequently dies without laying eggs (Nordmeyer and Sikora, 1983).

Effect of fungus and nematode infection on root nodulation

Leguminous root nodules are highly specialized structures formed as a result of a sequence of interactions between the host plant and the rhizobia. Root-nodule bacteria (*Rhizobium* and *Bradyrhizobium*) and leguminous plants show a mutually beneficial symbiotic relationship presenting a unique system of biological nitrogen fixation. This symbiotic association is of great agricultural importance. After the

period of nitrogen fixation, the mature nodules decays, liberating motile bacteria in the soil which normally serve as a source of inoculum for the succeeding crop of a given species of legume (Subbarao, 1975).

Generally antagonistic interaction between wilt fungus and root nodule forming bacteria have been recorded. Infection with wilt fungus causes suppression in nodulation (Twng-Wah and Howard, 1969; Swada, 1982, 83) but the mechanism involved is not properly determined. It appears that *Fusarium* infected roots due to physiological and structural modifications are rendered unsuitable for the development of root nodules. The suppression may also be due to competition between the two microorganisms at initial stage of the infection. Wilt causing fusaria are known to cause less infection on nodulated roots than non-nodulated roots (Zambolin and Schenk, 1984). Relationship between nematodes and root nodule forming bacteria is also negative. Plant parasitic nematodes also affect this system at various stages from its establishment to efficient functioning. Survival of root nodule bacteria in the rhizosphere and colonization in the rhizoplane are influenced by root exudates of nematode infected plants (Huang, 1987). Most investigators have reported that plant nematodes irrespective of their mode of parasitism inhibit nodulation. Nutrient depletion by the nematodes (Masefield, 1958), competition between nematode juveniles and root nodule bacteria (Epps and Chambers, 1962), devitalization of root tips (Malek and Jenkins, 1964) and suppression of lateral root formation (Oteifa and Salem, 1972) are possible causes of reduced nodulation. Haung *et al.* (1984) suggested that reduced nodulation on soybean resulted from interference of the nematode, *Heterodera glycines* with soybean lectin metabolism. The nematodes reduced the binding of rhizobia to infected roots.

Nematodes also damage root nodules by direct invasion. Species of *Meloidogyne, Heterodera* and *Pratylenchus* etc. invade root nodules directly on legumes. *Meloidogyne* spp. induce histological changes in nodular tissues and giant cells develop inside the nodules (Robinson, 1961; Barker and Hussey, 1976). Nodules also develop on root galls induced by the nematode (Khan and Kounser, 2000). Reduction in size and number of nodules and early degeneration of nodules on nematode infected leguminous plants are considered as two possible reasons for adverse effects on the nitrogen fixing capability of the roots (Khan *et al.*, 2002). Huang and Barker (1983) found a lower leghaemoglobin (Lb) content of nodules in soybean plants infected with *H. glycines* than uninfected plants. Chahal and Chahal

(1988) also reported significant reduction in Lb, bacteroid content and nitrogenase activity in chickpea nodules on roots infected with *M. incognita.*

Some investigations have indicated that nodulation in legume roots may be stimulated by nematode infection. Infection by *M. incognita, M. hapla, Pratylenchus penetrans* and *Belonolaimus longicaudatus* stimulated nodulation by *Bradyrhizobium japonicum* on soybean (Huang, 1987). *M. incognita* enhanced root nodulation on pea and black beans (Verdejo *et al.,* 1988). Some reports, however, show no apparent effect of nematode infection on root nodulation. Infection of *M. javanica* on cowpea (Taha and Kassab, 1980) and of *M. hapla, P. penetrans* and *B. longicausatus* on peanut (Barker and Hussey, 1976) did not influence nodule formation and their development.

Disease management

Plant diseases continue to threaten crop production in modern agriculture, thus challenging plant pathologists for their efficient management. In view of the prevalence of the diseases and the enormity of yield losses they cause to legume crops, considerable efforts have been made towards the management with varied success. The disease management can be accomplished by adopting chemical or non-chemical methods.

Chemical methods

By the need based use of chemicals and undertaking the steps to safeguard the user and environment against any possible health hazard, chemicals provide the most reliable means of disease control (Vyas, 1993). A number of fungicides like thiram, captan, carbendazim etc. are available in market and control plant diseases satisfactorily (Nene and Thapliyal, 1993). Treatment of seeds with fungicides before sowing ensures better plant stand and protects the chickpea crop from wilt infection in the early growth stage (Gurha *et al.*, 2003). Seed-borne inoculum of *F. oxysporum* f. sp. *ciceri* may be eradicated by treating seeds with 0.15 % Benlate T (30% benomyl + 30% thiram) @ 2.5 g/kg seed or Bavistin (carbendazim) @ 2.5 g/kg seed (Haware *et al.*, 1978). In green house tests, seed treatment with Bavistin or carboxin (0.25%) protected the plants infested with *F. oxysporum* f. sp. *ciceri* (Verma, 1976). Seed treatment with Bavistin + thiram (0.5 % + 2 g/kg seed) has also been found promising (Jalali *et al.*, 1980). Bavistin treatment (0.5 g/kg seed) improved seed

germination by 16.5%, greatly reduced wilt incidence and increased yield by 23.7% (Shukla *et al.*, 1981). Seed inoculation with *Rhizobium* followed by seed treatment with Bavistin (0.1%) is more effective in reducing wilt, increasing nodules/plant and yield than Bavistin (0.1%) alone (Anonymous, 1983). Rovral (0.2%), Mildothane (0.1%), Brassicol (0.2%) and Dithane M-45 (0.2%) treatments enhances seed germination and/or seedling vigour; Mildothane exhibited best responses (Shrisat and Kale, 1979). Mani and Sethi (1984) noted the influences of seed treatment with either Benlate, thiram, carbofuran or Oftanol on seedling emergence of chickpea in the presence of *Meloidogyne incognita*, *F. oxysporum* f. sp. *ciceri* and *F. solani*. Benlate and carbofuran gave the highest seedling emergence (69% and 64% respectively) as compared to controls (46%). Wilt incidence on tomato was effectively decreased by the application of carbendazim or benomyl (Soil drench) and consequently increased the yield. Carbendazim was found significantly superior to benomyl (Sen and Kapoor, 1974). Soil drench @ 400-10,000 ppm effectively manages the incidence of chickpea wilt (Shukla and Misra, 1972). Seed treatment by carbendazim @ 2 g/kg seed checked the wilt severity of chickpea by 51% (Khan *et al.*, 2004). Since the chickpea crop is principally grown in rainfed areas, many of the known conventional chemical methods have not found wide adoption (Jalali and Chand, 1992).

The methyl bromide treatment significantly reduced root galling and eggmass production of root-knot nematodes compared to other treatments in all crop cycles (Tzortzakakis and Petsas, 2003). Seed soaking of monocrotophos 40 EC @ 0.1% for 6 hours and soil application of carbofuran @ 2 kg/ha reduced root-knot disease of chickpea upto 34.8 and 51.8%, respectively. Grain yield of chickpea also increased, 8.0 and 11.8% due to monocrotophos and carbofuran, respectively (Patel *et al.*, 2005).

Disease management by cultural practices, soil amendments and use of resistant varieties

Cultural practices play an important role in integrated disease management. The losses caused by wilt may be curtailed by avoiding cultivation of chickpea in a heavily infested soil. Deep ploughing in hot summer months and removal of host plant debris from the field help in reducing the *F. oxysporum* f. sp. *ciceri* population in the soil

(Gurha *et al.*, 2003). *Fusarium* wilt and root rot in chickpea and pigeonpea can be effectively managed by deep ploughing and exposing the soil to sun in summer (Dhar, 2003). Other cultural practices such as delayed and deep sowing and soil amendment with oilseed cakes are also reported to reduce wilt incidence in fields (Singh and Singh, 1984; Dahiya *et al.*, 1988). Soil solarization (covering soil with transparent 100mm thick polythene sheet for 6-8 weeks from April to May) decreased population of *Fusarium* and plant parasitic nematodes that can attack chickpea (Chauhan *et al.*, 1998).

Use of disease resistant varieties is the safest and easy method to grow disease free crop. Butler (1918) had remarked that "the only thoroughly statisfactory way of dealing with wilt disease is the selection or breeding of resistant varieties." Some of the wilt resistant varieties of chickpea are CPS-1, Avrodhi, BG 209, Annegiri-1 etc. Development of improved and resistant varieties is a contineous process because of pathogenic variability and other factors that affect the resistance across locations and over time.

Reduction in nematode population has been found many fold by adapting some of the common cultural practices like flooding and fallowing, trap crops, cover crops, crop rotation etc. (Brown and Kerry, 1987; Kanwar and Bhatti, 1992). The use of sesame (*Sesamum indicum*) as intercrop in soil infested with *M. incognita* reduced the nematode population by 60% with tomato and 50% with sweet potato (Fernandez *et al.*, 1992). Amendment of soil with sawdust, urea, cowdung, leafmold, castor, mustard and neem cakes checked the development of *M. incognita* in tomato. A mixture of sawdust with different oil cakes improved the plant growth also (Singh *et al.*, 1985). Janathan *et al.* (2000) determined that soil amended with presmud @ 15 t/ha and neem cake 1.5 t/ha controlled root-knot (*M. incognita*) and increased plant height and yield of banana. Murugan (2001) conducted sand culture experiments with green gram, black gram, red gram and cowpea to evaluate effect of sawdust alcohol extract on root-knot caused by *M. incognita*. They found a drastic decrease in nematode population after the cultures were allowed to grow for 10, 20 and 40 days after inoculating the nematodes. Some manures like poultry manure, farm yard manure, goat manure and oil cakes of castor, neem, groundnut etc. satisfactorily controlled root-knot disease and increased the yield of plants (Alam and Jairajpuri, 1991; Dibakar *et al.*, 2003; Pokharel, 2000). Soil solarization is another soil disinfestation method for disease control. In Israel, Italy and USA, the use of 0.03

mm thick plastic transparent sheets in moist and well cultivated soil continuously for 30-50 days has been found to be effective against plant pathogenic fungi and nematodes including *M. incognita* in chickpea (Akem *et al.*, 2000).

The age old environment friendly disease management practices like sanitation, crop rotation, mixed cropping, date of sowing, fallowing, summer ploughing, green manuring, composting etc. to combat plant pathogens and the problem of soil sickness have gradually lost acceptability in the current farming system. The pace of development and durability of resistant varieties had been slow and unreliable in spite of tremendous advancements made in the genetic engineering. The chemical control too has its own limitations such as high capital investment, nonremunerative, poor availability, selectivity, temporary effect, efficacy affected by physico-chemical and biological factors, development of pest resistance, pest resurgence, pollution of food and feeds, health hazards, toxicity towards plants and animals, environmental contamination etc. Considering these limitations, there had been a growing awareness to devise management strategies, which alone or in integration with other practices could bring about a reasonably good degree of reduction in inoculum potential and/ or disease potential and at the same time ensure sustainability of the production, cost effectiveness and healthy ecosystem. Considering various prospects and consequences of the existing methods of disease management, the biological management is a system that appears to meet our needs in greater proportions with regard to economic viability and environmental sustainability.

Biological control

There is a developing consensus that chemical based farming is non-sustainable; as a result, more ecological approaches are now being researched. The most obvious and apparent environment friendly alternative to pesticides is to use naturally occurring biological approaches, to manage agriculturally important pests and diseases. Thus the term biological control came into existence. Way back in 1965 Garret defined biological control as "any condition under which, or practice whereby, survival and activity of a pathogen is reduced through the agency of any other living organism (except man himself) with the result that there is a reduction in the incidence of disease caused by the pathogen". Later on Baker and Cook (1974) defined biological control as the "reduction of inoculum density or disease producing

activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonists, or by mass introduction of one or more antagonists". Subsequently in 1983 they revised the definition to 'biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by one or more organisms other than man'. Biological control can be achieved either by introducing biocontrol agents directly into natural ecosystem or by adopting practices which favour population build up of biocontrol agents under natural condition. In 1874, W. Roberts demonstrated for the first evidence of antagonistic action of microorganisms in liquid cultures between *Penicillium glaucum* and a bacterium and introduced word 'antagonism' (Baker, 1987). Since then, a lot of data has been generated to prove that biological control is a realistic proposition for disease management. The first attempt at control of a root disease with microorganims introduced into the soil was by C. Hartley in 1921 where introduction of isolates of saprophytic fungi and one bacterium gave significant reduction in severity of damping-off of pine seedlings caused by *Pythium debaryanum* (Baker, 1987). Parasitic nature of *Trichoderma lignorum* on several plant pathogens, including *Rhizoctonia solani*, *Phytopthora parasitica*, *Pythium* spp., *Sclerotium rolfsii* was reported for the first time by R. Weindling in 1932. The report on the possible use of mycoparasites for the control of plant pathogens aroused immense interest in the area of biological control of plant pathogens (Boosalis, 1964; Baker and snyder, 1965; Mukhopadhyay and Mukherjee, 1998).

In India, Vasudeva and Roy (1950) did pioneering work on the management of pigeonpea wilt caused by *Fusarium udum*. They explained the low incidence of pigeonpea wilt due to suppression of the pathogen by an antagonistic bacterium *Bacillus subtilis*, which released some antibiotics in the rhizosphere. A medium was developed for the increased production of this antibiotic. The antibiotic produced by *B. subtilis* was isolated and named as bulbiformin (Vasudeva *et al.*, 1958). Later on this bulbiformin was detected in the rhizosphere indicating that *B. subtilis* produced bulbiformin in the soil. Under conditions favourable for antibiotic production, *B. subtilis* brought about a marked decrease in the incidence of pigeonpea wilt (Vasudeva *et al.*, 1962). Mukhopadadhyay did pioneering work on biological control of important soil-borne fungi. Upadhyay and Mukhopadadhyay (1986)

demonstrated the suppression of *Sclerotium* root rot of sugar beet by nitrogenous fertilizers, which was due to increased resident population of *Trichoderma harzianum* in soil following application of these fertilizers. Later Jayaraj and Ramabadran (1998a, b) also demonstrated favourable effect of nitrogenous fertilizers on growth , sporulation and survival of *T. harzianum* both under *in vitro* and in soil.

Biocontrol fungi

Trichoderma species

Division	Ascomycota
Sub division	Pezizomycotina
Class	Sordariomycetes
Order	Hypocreales
Family	Hypocreaceae
Genus	*Trichoderma*

Mechanism of disease suppression

The activities of biocontrol agents mainly depends on different physicochemical environmental conditions to which they are subjected. Understanding both the genetic diversity of strains within *Trichoderma* species and their mechanisms of biocontrol will lead to improved application of the different strains as biocontrol agents. These mechanisms are complex, and what has been defined as biocontrol is the final result of different mechanisms acting synergistically to achieve disease control (Howell, 2003). Biocontrol results either from competition for nutrients and space or as a result of the ability of *Trichoderma* biocontrol agents to produce and/or resist metabolites that either impede spore germination (fungistatis), kill the cells (antibiosis) or modify the rhizosphere, e.g. by acidifying the soil, so that pathogens cannot grow. Biocontrol may also result from a direct interaction between the pathogen itself and the biocontrol agent, as in mycoparasitism, which involves physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes. *Trichoderma* spp. can even exert positive effects on plants with an increase in plant growth (mineralization) and the stimulation of plant defense mechanisms. Mechanism of disease suppression may be due to competition, antibiosis or mycoparasitism.

Competition

Fungistatis: The nature of competition is fungistatic (inhibitor). Good antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites produced by other species including plants and to survive under very extreme competitive conditions.

Trichoderma strains grow rapidly when inoculated in the soil because they are naturally resistant to many toxic compounds including herbicides, fungicides and pesticides such as DDT and phenolic compounds (Chet *et al.*, 1997). Resistance to toxic compounds may be due to the presence of ABC transport systems in *Trichoderma* strains (Harman *et al.*, 2004). *Trichoderma* strains are very efficient in controlling several phytopathogens such as *R. solani*, *P. ultimum* and *S. rolfsii* when alternated with methyl bromide, benomyl, captan or other chemicals due to the presence of ABC transport system (Vyas and Vyas, 1995).

Competition for nutrients: Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Chet *et al.*, 1997). For instance, in most filamentous fungi iron uptake is essential for viability (Eisendle *et al.*, 2004) and under iron starvation most fungi excrete low molecular weight ferric iron specific chelators termed as siderophores to mobilize environmental iron (Eisendle *et al.*, 2004). For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* according to iron availability. Some *Trichoderma* biological agents produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet and Inbar, 1994). One of the most sensitive stages for nutrient competition in the life cycle of *Fusarium* is chlamydospore germination (Baker, 1986). In soil the chlamydospores of *F. oxysporum,* need nutrition to maintain a germination rate of 20-30%. The germination may decrease due to sharing of nutrients by other microorganisms. Root exudates are major source of nutrients in soil which are excreted from the root tips. Thus, colonization in the rhizosphere of root tip by an antagonist might reduce infection by *Fusarium*-like pathotypes (Cook and Baker, 1983). In addition, *T. harzianum* T35 controls *F. oxysporum* by competing for both rhizosphere colonization and nutrients with biocontrol becoming more effective as the nutrient concentration decreases (Tjamos *et al.*, 1992). Competition for carbon has also been involved in the determination of the antagonism

expressed by different strains of *Trichoderma* spp. against several plant pathogens, especially *F. oxysporum* (Sivan and Chet, 1989). *T. viride* controlled *Chondrostereum purpureum,* the silver leaf pathogen of plum trees due to competition exerted by the former (Corke and Hunter, 1979). Competition has proved to be particularly important for the biocontrol of phytopathogens such as *Botrytis cinerea*, the main pathogenic agent during the pre and post-harvest in many countries (Latorre *et al.*, 2001). The advantage of using *Trichoderma* to control *Botrytis cinerea* is the coordination of several mechanisms, the most important is nutrient competition, since *Botrytis cinerea* is particularly sensitive to the lack of nutrients.

Trichoderma has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide spread in fungal environments: cellulose, glucan and chitin among others, all of them rendering glucose (Chet *et al.*, 1997). While the role of the glucose transport systems remains to be discovered, its efficiency may be crucial in competition (Delgado-Jarana *et al.*, 2003) as supported by the isolation of a high affinity glucose transporter, Gtt 1, in *T. harzianum* CECT 2413. This strain is present in environments very poor in nutrients, and it relies on extracellular hydrolases for survival. The Gtt 1 is only expressed at very low glucose concentrations, i.e., when sugar transport is expected to be limiting in nutrient competition (Delgado-Jarana *et al.*, 2003).

Antibiosis

Antibiosis is required as one of the most important attribute in deciding the competitive saprophytic ability of *Trichoderma* spp. Antibiosis occurs during interactions involving low molecular weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. Most *Trichoderma* strains produce volatile and non volatile metabolites (Table 9) that impede colonization by antagonized microorganisms; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-a-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described (Vey *et al.*, 2001). In some cases, antibiotic production correlates with biocontrol ability, and purified antibiotics mimic the effect of the

Table 9. Antibiotics or antibiotics-like effectors produced by *Trichoderma* species.

Antibiotics or antibiotics-like effectors	Reference
Trichodermin	Godtfredson and Vangedal, 1965
Trichoviridin	Yamano *et al.*, 1970
Trichosetin	Marfori *et al.*, 2002
Gliotoxin, Trichodermin, Viridin	Haggag and Mohamed, 2002
Chitinase	Elad *et al.*, 1982
Protease	Elad *et al.*, 2000b
Chitobiase	Ulhao and Peberdy, 1993
Sesquiterpene heptalic acid	Itoh *et al.*, 1980
α-gluxosidase protein	Shanmugam *et al.*, 2001
Dermadin	Pyke and Dietz, 1966
β-1,3-glucanase	Perez *et al.*, 2001,
Alamethicine, Paracelsin, Trichotoxin	Lumsden *et al.*, 1991
Heptelidic acid	Howell *et al.*, 1993
Chitin-1-4-β-chitobiosidase n-acetyl, β-D glucosaminase, Endochitinase	Harman *et al.*, 1993
6-n-pentenyl-2H-pyran-2 one, 6-n-pentenyl-2H-pyran-2-one,	Claydon *et al.*, 1987
Harzianolide [3-(2-hydroxyl-propyl) -4(hexa-2"-dienyl-2(5H) furanone	Claydon *et al.*, 1991
Trichorzianines, Trichorviridin, Propionic acid, 3-(3-isocyanocyclopent-2-enzylidene), Acrylic acid, 3-(3-isocyano-6-oxabicyclo (3, 10) hex-2-eh-5-yl	Baldwin *et al.*, 1981

whole agent. Volatile substances from *Trichoderma* spp. inhibited the mycelial growth of *Macrophomina phaseolina* by 22-51% (Angappan, 1992). The volatile antibiotics of *T. harzianum* and *T. atroviride* significantly decreased the growth of canker pathogen fungi of poplar, *Cytospora chrysosperma* and *Dothiorella gregaria* (Gao *et al.*, 2001). Non-volatile metabolites in the culture filtrate of *Trichoderma* spp. inhibited the linear growth of pathogens (Deshmukh and Pant, 1992; Pande, 1985). Dwivedi (1992) reported that culture filtrate of *T. harzianum* inhibited the growth of *F. solani* and *F. longipus* by 60 and 64%, respectively.

There are also examples of antibiotic overproducing strains such as gliovirin overproducing mutants of *T. virens*, which provide control similar to that of the wild type and of gliovirin deficient mutants which failed to protect cotton seedlings from *Pythium ultimum*, whereas the parental strain did (Chet *et al.*, 1997). In general, strains of *T. virens* with the best efficiency as biocontrol agents are able to produce gliovirin (Howell, 1998). Also, the most effective isolates of *T. harzianum* against *Gaeumannomyces graminis* var. *tritici* produce pyrone antibiotics and the success of the strains was clearly related to the pyrones they produced (Benitez *et al.*, 2004). *Trichoderma* spp. are reported to produce carbon monoxide, ammonia (Dennis and Webster, 1971b), carbomyl compounds and acetaldehyde (Robinson and Park, 1966) that may enhance the antagonistic activity in soil. Similar results were obtained against apple ring rot pathogen, *Botryosphaeria berengeriana* f. sp. *piricola* (Gao, 2002). The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Howell, 1998; Monte, 2001). Synergetic effects between an endochitinase from *T. harzianum* and gliotoxin, and between hydrolytic enzymes and peptaibols on conidial germination of *B. cinerea* is well known (Howell, 2003). A mutant from strain *T. harzianum* CECT 2413 that had higher levels of extracellular enzymes and of a-pyrone performed better than the wild type in *in vitro* experiments against *R. solani* and in assays of grape protection against *B. cinerea* (Rey *et al.*, 2001).

Mycoparasitism

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events including recognition, attack and subsequent penetration and killing of the host. *Trichoderma* spp. may exert direct biocontrol by parasitizing a range of fungi detecting other fungi and growing towards them. The

remote sensing is partially due to the sequential expression of cell wall degrading enzymes, mostly chitinases, glucanases and proteases (Harman *et al.*, 2004). Mycoparasitism involves morphological changes, such as coiling and formation of appressorium like structures which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol (McIntyre *et al.*, 2004). *Trichoderma* attaches to the pathogen with cell wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria. The following step consists of the production of cell wall degrading enzymes and peptaibols (Howell, 2003) which facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell wall content. *Trichoderma* spp. react violently with hyphae of the *Fusarium* species. The hyphae of *Trichoderma* spp. when near to pathogen induce morphological deformalities in the host hyphae. Many a time bursting of hyphae and vacoulation has been observed (Komatsu, 1968; Gao *et al.*, 2001). In addition, granulation, coagulation, disintegration and finally lysis of the pathogen occurs (Lim and Teh, 1990; Elad *et al.*, 1983; Nigam *et al.*, 1997; Gao, 2001). *In vitro* studies have revealed that purified endochitinase, chitobiosidase, n-acetyl-b-glucosidase and glucan 1,3-β-glucosidase, and combinations thereof, greatly suppressed the spore germination and germ tube elongation in nine different fungal species (Lorito *et al.*, 1993; Di Pietro *et al.*, 1993; Lorito *et al.*, 1994a, b). *T. harzianum* TM transformants overexpressing chit36 chitinase inhibited *F. oxysporum* and *S. rolfsii* more strongly than the wild type. Moreover, culture filtrates inhibited the germination of *B. cinerea* almost completely (Viterbo *et al.*, 2001).

Stimulation of host defence response

The ability of *Trichoderma* strains to protect plants against root pathogens has long been attributed to an antagonistic effect against the invasive pathogen (Chet *et al.*, 1997). However, these root fungus associations also stimulate plant defensive mechanisms (Howell *et al.*, 2000; Hanson and Howell, 2004). Strains of *Trichoderma* added to the rhizosphere protect plants against numerous classes of pathogens, e.g. those that produce aerial infections, including viral, bacterial and fungal pathogens, which point to the induction of resistance mechanisms similar to the hypersensitive response (HR), systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants (Harman *et al.*, 2004). At a molecular level,

resistance results in an increase in the concentration of metabolites and enzymes related to defensive mechanisms such as the enzymes phenyl alanine ammonia lyase (PAL) and chalcone synthase (CHS), involved in the biosynthesis of phytoalexins (HR response), chitinases and glucanases. These comprise pathogenesis related proteins (SAR response) and enzymes involved in the response to oxidative stress. The addition to *Trichoderma* metabolites that may act as elicitors of plant resistance or the expression in transgenic plants of genes whose products act as elicitors, also results in the synthesis of phytoalexins, PR proteins and other compounds and in an increase in resistance against several plant pathogens, including fungi and bacteria (Dana *et al.*, 2001; Elad *et al.*, 2000a) as well as resistance to hostile abiotic conditions (Harman *et al.*, 2004). Barley expressing *Trichoderma atroviride* endochitinase Ech 42 showed increased resistance towards *Fusarium* infection (McIntyre *et al.*, 2004). Cotton seedlings treated with efficient strain of *T. virens* had higher levels of defense related compounds such as terpenoids and peroxidase activity in the root (Howell *et al.*, 2000). An ethylene-inducing xylanase produced by *T. viride* (Dean and Anderson, 1991) elicited the production of phytoalexin reversatrol in grapevine cells (Calderon *et al.*, 1993). Hanson and Howell (2004) reported that culture filtrates from a strain of *T. virens* stimulated synthesis of terpenoid in cotton and the elicitors were presumably proteins or glycoproteins.

Plant growth promotion by *Trichoderma* species

Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Arora *et al.*, 1992). Crop productivity in fields can increase upto 300% after the addition of *T. hamatum* or *T. koningii.* The experiments carried out in green houses with seed treatment with *Trichoderma* spores have shown significant greater yield (Chet *et al.*, 1997). Equal degree of yield enhancement was observed when plant seeds were separated from *Trichoderma* by a cellophane membrane. This indicates that *Trichoderma* produces growth factors that enhanced the rate of seed germination, plant growth and yield (Benitez *et al.*, 1998). Zimand *et al.* (1996) reported that *T. harzianum* T39 besides having inhibitory effect on the conidial germination and germ tube elongation of *Botrytis cinerea,* also reduced the production and activity of pathogen secreted pectolytic enzymes three days after inoculation. Reduced activities of pectolytic enzymes may increase the accumulation

of pectic enzyme products i.e., oligogalacturonides. These sugars can elicit the host plant (bean) defence mechanisms, thus checking the disease development. Activity of biocontrol agents could also reduce the concentration of substances in soil that are inhibitory to plant growth (Windham *et al.,* 1986). Thus, the plant growth promotion may be due to production of plant hormones or increased uptake of nutrients by the plant (Chet *et al.*, 1993); control of one or more sub potential pathogens (Baker, 1986) and/or strengthening plant's own defense mechanism (Zimand *et al.,* 1996).

Application of *Trichoderma* spp. against pathogenic fungi

Coating seeds with biocontrol agent *T. harzianum*, *T. viride* and *T.* (*Gliocladium*) *virens* significantly controlled *Fusarium oxysporum* f. sp. *ciceri* wilt by 30-46% and integration of biocontrol agent and carboxin increased the seed yield by 25-43% (De *et al.*, 1996). Chickpea and lentil seeds treated with *T. virens* (10^7 conidia/ ml) and then with 0.1 percent carboxin effectively reduced soil-borne population of *F. oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* (Mukhopadhyay *et al.*, 1992). Soil application of *T. harzianum* gave 54 – 86% disease control in a glass house study. In the field, integrated use of *T. harzianum* with fungicidal seed treatments significantly reduced the incidence of chickpea wilt complex and increased crop yield. Seed treatment with vitavax and ziram resulted to 30% disease control, the control, however, increased to 63% when *T. harzianum* was also applied (Kaur and Mukhopadhyay, 1992).

Trichoderma harzianum was found antagonistic *in vitro* to *R. solani* and *V. dahliae* at 15-25°C and inhibited the development of *R. solani* and *V. dahliae* at both the temperatures (Santamarina and Rosello, 2006). The application of *T. harzianum* formulation based on tea leaves and wheat bran sawdust significantly reduced the percent mortality due to chickpea wilt complex and groundnut collar rot disease, respectively. Population count after 30 days was maximum in used tea leaves (8×10^8 cfu/g) and shelf life was found to be maximum (2.9×10^5 cfu/g after 210 days) in wheat bran sawdust (Singh *et al.*, 2006). *Trichoderma* spp. significantly reduced stem canker and black scurf diseases of potato (Brewer and Larkin, 2005). A significantly long period of protection from *P. expansum* infection (upto 2 months) was obtained when unwounded apple fruits were dipped for 30 seconds period in formulated *T. harzianum* conidia before being inoculated by *P. expansum* compared

to the wounded fruits (Batta, 2004). Coating chickpea seeds with biocontrol agents like *T. virens*, *T. harzianum* and *T. viride* significantly controlled *F. oxysporum* f. sp. *ciceri* by 30-46% (De-Souza *et al.*, 2003). Kaur and Mukhopadhyay (1992) reported that the soil application with *T. harzianum* gave 54 to 88% control of fusarium wilt of chickpea in glasshouse.

Bean seeds sown in heavily infested soil and treated with conidia of the transgenic *Trichoderma* strain germinated, but with wild type spores did not germinate (Brunner *et al.*, 2005). Transgenic strain SJ3-4 of *T. atroviride* not only exhibited threefold greater potential of inhibition of spore germination of *Botrytis cinerea* but also overgrew and caused lysis to *Rhizoctonia solani* and *Pythium ultimum* (Brunner *et al.*, 2005). Chitinase production and bioactivity of 15 self fusants of *T. harzianum* were studied. The fusant strain SFTh8 produced maximum chitinase with a two fold increase as compared to the parent strain. All the self fusants exhibited increased antagonistic activity against *R. solani* (Prabavathy *et al.*, 2005). In our field *T. harzianum* causes 30 and 60% decrease in incidence and severity of wilt in chickpea as compared to control (Khan *et al.*, 2004). Application of *T. harzianum* provided the highest control of *F. oxysporum f. sp. ciceri* causing wilt in chickpea both *in vitro* and under field conditions (Singh, *et al.*, 2003). *T. harzianum* had better antagonistic efficiency against 10 isolates of *F. oxysporum f. sp. ciceri* compared to *T. viride* (Gurha, 2001). Prasad *et al.* (2002) evaluated *T. harzianum* PDBCTH 10 and *T. viride* PDBCTV against natural incidence of chickpea wilt. The wilt incidence was higher (12 and 16%) in control plots, but in *T. harzianum* soil treated plots only 4 and 5.1% wilt incidence was observed at 60 and 90 days, respectively.

Singh and Singh (2004) reported that *T. harzianum* controlled the *S. rolfsii* the causal agent of collar rot in mint by 67-100%. Khan and Akram (2000) observed significant decrease in the wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* by soil application of *T. virens.* Application of *T. harzianum* considerably checked the root-rot of chickpea caused by *R. solani* and subsequently the yield of tested chickpea varieties increased by 40-65% (Khan and Rehman, 1997). Khan and Gupta (1998) recorded *T. harzianum, T. hamatum* and *T. viride* most effective out of five species of *Trichoderma* tested against *Macrophomina phaseolina* on egg plant in a field. Application of *T. koningii, T. hamatum* and *T. virens* greatly suppressed the growth of *F. oxysporum* f. sp. *lycopersici* (Cipriano *et al.*, 1989). Seed treatment

with *T. harzianum* or *P. lilacinus* controlled the wilt of tomato (Shahida and Ghaffar, 1991). Jash and Pant (2004) observed that *T. harzianum* effectively controlled the root rot caused by *Rhizoctonia solani* in sterilized and nonsterilized soil. In dual culture the biocontrol agents inhibited the growth of the fungus by 74%. Culture filtrate of *Trichoderma* spp. have also been found inhibitory to pathogenic fungi. The culture filtrate of *T. harzianum* reduced the mycelial growth of the pathogens from 52-87% (Ngueko and Xu, 2002). *T. harzianum* strain C184 was tested for its antagonism against *Cylindrocladium pteridis* causing root necrosis in banana and plantain and *F. solani, F. oxysporum* and *Aspergillus* sp. which are secondary colonizers of root system on these crops . On dual culture the mycelial growth of the pathogens was significantly suppressed. *T. virens* strongly antagonized *Pythium aphanidermatum,* incitant of tomato damping-off. *T. virens* hyphae ran parallel to, entwine around, penetrated into and killed the host hyphae. Chaudhary and Prajapati (2004) reported *in vitro* antagonism of *T. harzianum* and *T. virens* against *F. udum.* The antagonists reduced the colony growth of *F. udum* through saprophytic competition. *T. harzianum* showed maximum growth in a dual culture test and effectively inhibited the growth of *Macrophomina phaseolina* (65%) (Malathi and Doraisamy, 2004). Similar effects of *T. harzianum* have also been reported on *R. bataticola* (Rajarkar *et al.,* 1998), *Sclerotium rolfsii* (Prasad *et al.,* 2003) and *F. udum* (Singh *et al.,* 2002). Isolates of *T. harzianum, T. virens* and *T. viride* were found to exhibit strong antagonism by inhibiting hyphal growth of *F. udum* in a dual culture test. *T. harzianum* caused severe vacoulation, shrinkage and coagulation of the cytoplasm of pathogen hyphae. *T. virens* caused twisting, air bubbling and disintegration whereas, *T. viride* formed loops and coils around the pathogen hyphae. After 9 days of incubation, lysis of hyphae, rupturing of the cell wall and leakage of the cytoplasm of the parasitized pathogen occurred (Pandey and Upadhayay, 2000).

Application of *Trichoderma* spp. against phytonematodes

Eapen and Venugopal (1995) have shown that isolates of *Trichoderma* spp. have a broad spectrum biocontrol activity against a number of pathogenic fungi and nematodes. A serine protease of 28 kDa with trypsin activity was isolated from *Trichoderma* strain 2413. The enzyme reduced the number of hatched eggs of root knot nematodes and showed synergistic effects with other proteins produced during antagonistic activity of the strain (Suarez *et al.*, 2004).

The number of hatched eggs of the root knot nematode, *Meloidogyne incognita* was significantly reduced after incubation with pure PRA1 (trypsin like protease) preparations of *T. harzianum* CECT 2413 (Suarez *et al.*, 2004). Hemlata *et al.* (2002) studied the effect of using *T. harzianum* and neem cake alone and in combination to manage *M. incognita* in chickpea cv. Type-3. Greatest reduction in the root-knot nematode was recorded with the application of neem cake and *T. harzianum* together, followed by neem cake and *T. harzianum* alone. Siddiqui and Shaukat (2004) reported that combined application of *T. harzianum* with *P. fluorescens* in unsterilized sandy loam soil caused greater reduction in *M. javanica* population densities in tomato roots. Strains of *T. virens* and *Burkholderia cepacia* (bacterium) were found to produce extracellular factors *in vitro* that decreased *M. incognita* egg hatch and juvenile mobility. In green house studies, individual application of these microorganism as seed coat followed by root drenches suppressed root-knot nematode population on bell pepper compared with untreated plants (Meyer *et al.*, 2001). Pant and Pandey (2001) reported maximum reduction in the population of *M. incognita* by *T. harzianum, P. lilacinum* and *A. niger* applied in sterilized soil in pots @ 5000 spores/pot. Significant reduction in *M. incognita* was also observed when *T. harziaum* was applied along with the neem cake (Pant and Pandey, 2002). Windham *et al.* (1989) tested the effect of *T. harzianum* applied before corn seeding, in small pots filled with *M. arenaria* infested soil; at harvest, after 50 days, top and root fresh weights had increased, with a decrease in the number of eggs per gram root, as compared to the control plants. Suppression of root-knot nematodes resulting in improved growth of cardamom seedlings in nurseries has been reported with *T. harzianum* (Anonymous, 1991; 1994b; 1995).

***Pochonia chlamydosporia* (=*Verticillium chlamydosporium*) (Goddard, 1913) Zare and Games, 2001**

Taxonomic Position

Division	Ascomycota
Class	*Incertae sedis*
Subclass	Hyphomycetidae
Genus	*Verticillium*

Mechanism of disease suppression

Pochonia chlamydosporia (= *Verticillium chlamydosporium*) is widespread in cyst and root-knot infested soils around the world (Kerry, 1993). All stages of the fungus (hyphae, conidia, chlamydosporium) occur in soil. The actively growing mycelium infects eggs and females of nematodes (Davies *et al.*, 1991). The fungus forms close contact with the egg shell and causes disintegration of eggshell, vitelline layer and partial dissolution of chitin and lipid layer (Lopez Llorca and Duncan, 1988; Saifullah and Thomas1997; Stirling, 1991). Egg hatching in the presence of fungus was inhibited probably due to toxins secreted by the fungus (Meyer *et al.*, 1990; Morgan-Jones *et al.*, 1983). The fungus produces an appressorium that adheres to the eggshell by mucigens and from which an infection peg develops that penetrates the eggshell (Seggers *et al.*, 1996). Penetration also occurs from lateral branches of the mycelium. It has been observed that the fungus causes disintegration of eggshell's vitelline layer and also partial dissolution of the chitin and lipid layers, possibly due to the activity of exoenzymes. Serine proteases have been identified in *P. chlamydosporia* (Seggers *et al.*, 1994). These extracellular enzymes are synthesized in the presence of nematode eggs and repressed by glucose. There is much variation in the subtilisins produced by different isolates of *P. chlamydosporia* (Seggers *et al.*, 1999). In addition to the direct effects of parasitism by *P. chlamydosporia* on the developing embryo, enzymatic effects on the eggshell may increase permeability and possibly facilitate the inward passage of toxins that may have been present in the environment. It has been suggested that *P. chlamydosporia* itself might produce such a toxin, because eggs did not hatch when the fungus was near the eggs (Morgan-Jones *et al.*, 1983; Meyer *et al.*, 1990). However, Irving and Kerry (1986) obtained no evidence to support toxin production.

Under natural soil conditions, nematode eggs appear to be an important source of nutrients for *P. chlamydosporia.* The fungus parasitizes large number of *H. avenae* eggs in english cereal fields and played a major role in limiting multiplication of nematode (Kerry *et al.*, 1982a, b). *P. chlamydosporia* does not depend on nematodes for its nutrition as it has been isolated from oospores of some fungi (Sneh *et al.*, 1977) and snail eggs (Barron and Onions, 1966). The fungus also colonizes plant roots (Thornton, 1965; Kerry *et al.*, 1984) and degrades both cellulase and chitin (Domsch *et al.*, 1980). Thus, *P. chlamydosporia* is an unspecialized, opportunistic species with the capacity to compete for many available substrates in

soil including nematodes. Isolates of *P. chlamydosporia* vary greatly in growth, sporulation, temperature requirements and chlamydospore production, while some strains are relatively weak pathogens, others are aggressive parasites (Kerry, 1981; Kerry *et al.*, 1986; Irving and Kerry, 1986). The presence of root-knot nematodes in roots, however, results in increased densities of *P. chlamydosporia* in the rhizosphere (Bourne *et al.*, 1996). Although galled roots were most extensively colonized by *P. chlamydosporia,* the fungus was least effective in controlling *M. incognita* at high nematode densities, presumably because many egg masses stayed embedded in the gall tissue and were, therefore, protected from fungal attack. Hence, control with this agent is minimal unless the fungus is present on relatively poor hosts for the nematode on which only small galls are produced and most egg masses are exposed on the gall surface. The fungus also produces nematicidal metabolites. Culture filtrate of *P. chlamydosporia* in yeast extract has caused nematicidal and nematostatic effects. A dilution of 1:1 culture filtrate caused 100% mortality of *G. rostochiensis, G. pallida* and *Panagrellus redivivus* (Saifullah, 1996c).

Application of *Pochonia chlamydosporia* against phytonematodes

The nematophagous fungus, *P. chlamydosporia* var. *catenulate* was investigated as a potential biocontrol agent in integrated management strategy for *M. incognita* in vegetables in Cuba (Garcia *et al.*, 2004). The presence of *P. chlamydosporia* was associated with a reduction in the number of plant parasitic nematodes (51-78%) including the migratory ectoparasites, whereas free living nematodes, culturable bacteria and bacterial populations assessed by Biolog were unaffected by the application of fungus (Tahseen *et al.*, 2005). Olivares and Lopez Llorca (2002) investigated the presence of fungal egg parasites in Spanish soils with plant endoparasitic nematodes. The most common was *P. chlamydosporia* var. *chlamydosporia* causing (70-100% egg infection) and severity (35-40 penetrating hyphae/egg) on *M. javanica.* Application of *P. chlamydosporia* decreased the number of eggs, juveniles and galls in tomato plants grown in pots containing an unsterilized peat/sand/compost mixture (De Leij *et al.*, 1992). Soil population of *M. hapla* in tomato root zone decreased by more than 90% in sandy loam soil applied with *P. chlamydosporia* alone or with aldicarb (De Leij *et al.*, 1993). Bhardwaj and Trivedi (1996) reported significant reduction in *H. avenae* populations and increase in wheat plant growth in *P. chlamydosporia* treated pots. The best

control of *M. javanica* was obtained by inoculating the soil with *P. chlamydosporia* colonized on rice medium at the rate of 30 g/kg soil. Introduction of the fungus two weeks before the nematode inoculation provided significantly greater control of *M. javanica.* Organic amendments with castor, marigold and neem stimulated the parasitism by *P. chlamydosporia,* while benomyl and mustard caused inhibitory effects (Owino *et al.,* 1993). Kerry and Bourne (1996) suggested that *P. chlamydosporia* in conjunction with other methods, such as rotation of the poor host, may provide adequate control but the reliability of such approaches needs extensive testing. Cannayane and Rajendran (2001) reported that application of *P. chlamydosporia* @ 20 g/plot (6 x 10^7 CFU/g substrates) along with *P. lilacinus* and neem cake effectively controlled the *M. incognita* and increased 58% yield of inoculated brinjal plants. Application of *P. chlamydosporia* and *T. harzianum* were found to parasitize females (Saifullah, 1996a) and males (Saifullah, 1996b) of *Globodera rostochiensis* and *G. pallida.* Viaene and Abawi (2000) reported that *P. chlamydosporia* colonized egg masses of *M. hapla* in lettuce and the colonization varied from 16-43%. The fungus was found effective in reducing the *M. hapla* population at densities below 8 eggs/cm^3 soil. Siddiqui and Mahmood (1996) reported a combination of *P. chlamydosporia, T. harzianum* and *G. mosseae* caused highest reduction of *H. cajani* multiplication in pigeonpea. Kerry (1988) showed that hyphae of *P. chlamydosporia* grew approx. 1 cm from alginate granules, which suggest that such granular formulations may be suitable for this species. Coosemans (1988) reported establishment of *P. chlamydosporia* in field soil after it was grown on a substrate and incorporated into a peat growth medium. Galling caused by root-knot nematode was substantially reduced when *P. chlamydosporia* grown on oat kernels was introduced into field soil (Godoy *et al.,* 1993; Rodriquez-kabana *et al.,* 1984). Khan *et al.* (2001) tested a variety of agricultural materials and wastes to rear the fungus. They recovered greater colonization on compost, leaflitter and sawdust.

Bacterial biocontrol agents

The use of bacteria as a suppressant of plant pathogenic fungi has been investigated mainly because genetic and biochemical analyses and the mass production of bacteria or bacterial products are much easier than those of fungi, and thus bacterial control is expected to have greater potential (Shoda, 2000). As bacterial biocontrol agents,

Agrobacterium, *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Streptomyces* and others have been reported but usefulness of certain species of *Bacillus* (Campbell, 1989) and *Pseudomonas* (Wei *et al.*, 1996) as antagonist for many plant pathogens is well known (Table 10).

***Bacillus subtilis* (Cohn) Prazmowski**

Group	Gram positive endospore forming rods and cocci
Family	Bacillaceae
Genus	*Bacillus*
Species	*subtilis*

***Pseudomonas fluorescens* (Threvesan) Migula**

Group	Gram negative aerobic rods and cocci
Family	Pseudomonadaceae
Genus	*Pseudomonas*
Species	*fluorescens*

Mechanism of disease suppression

Different mechanisms are involved in the actions of these microorganisms against plant pathogens. The chief modes of action are as follows:

Rhizosphere competence and colonization

Rhizosphere competence describes the relative root colonizing ability of a rhizobacteria (Weller and Thomashow, 1994). A key feature of all rhizobacteria is that they all colonize roots to some extent. In some cases this may involve specific attachment for example, pili, as with the attachment of *Pseudomonas fluorescens* 2-79 to the surface of wheat roots (Vesper, 1987). However, such specific attachment does not seem to be an absolute requirement for colonization (de Weger *et al.*, 1995). Colonization may involve simply root surface development but endophytic colonization of the root is also known and the degree of endophytic colonization depends on bacterial strain and plant type. Endophytic growth in roots has been recorded with *Bacillus polymyxa* Pw-ZR and *Pseudomonas fluorescens* Sm3-RN on spruce (Shishido *et al.*, 1999), with the biocontrol strains of *Bacillus* sp. L324-92R_{12} and *Pseudomonas fluorescens* 2-79RN_{10} on wheat (Kim *et al.*, 1997b) and

Table 10. *Bacillus* and *Pseudomonas* species as bio-control agents of plant diseases.

Bioagent	Pathogen	Reference
Pseudomonas spp.	*Fusarium* spp.	Chen *et al.*, 1995
Pseudomonas spp.	*Pythium* spp.	Paulitz *et al.*, 1992
P. fluorescens	*Fusarium oxysporum*	Nautiyal, 1997
P. fluorescens, P. putida	*Sclerotinia* spp.	Expert and Digat, 1995
P. fluorescens	*F. o.* f. sp. *ciceri*	Khan *et al.*, 2004
P. fluorescens	*Aspergillus, Curvularia, R. solani*	Sindhu *et al.*, 1999
P. glumae	*R. solani*	Pal, 1995
Bacillus spp.	*Botrytis cinerea*	Sharga, 1997
Bacillus spp.	*F. avenaceum*	Hwang, 1994
B. subtilis	*R. solani*	Asaka and Shoda, 1996b
B. cereus	*Phytophthora* sp.	Handlsman *et al.*, 1990
B. subtilis, P. fluorescens	*Fusarium oxysporum* f. sp. *lycopersici*	Khan and Khan, 2001

with several that induce resistance such as *Bacillus pumilus* SE34 and *P. fluorescens* 63-28 on pea (Benhamou *et al.*, 1996a, b; M' Piga *et al.*, 1997), and *P. fluorescens* CHAO on tobacco (Troxler *et al.*, 1997) and *P. fluorescens* WCS417r on tomato (Duijff *et al.*, 1997). Indeed, many seeds, roots and tubers are normally colonized by endophytic bacteria (McInroy and Kloepper, 1995; Sturz *et al.*, 1999).

Antibiosis

There are numerous reports of the production of antifungal metabolites (excluding metal chelators and enzymes) produced by bacteria *in vitro* that may also have activity *in vivo*. These include bacillomycin, (Peypoux *et al.*, 1980; Chevanet *et al.*, 1985), iturin (Delcambe *et al.*, 1977; Peypoux *et al.*, 1978), surfactin, mycosubtilin (Peypoux *et al.*, 1986), bacilysin (Roger *et al.*, 1965; Loeffler *et al.*, 1986) fengymycin (Roger *et al.*, 1965), mycobacillin (Majumandar and Bose, 1970), ammonia, butyrolactones, 2,4-diacetylphloroglucinol, HCN, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin and zwittermycin A as well as several other uncharacterized moieties (Milner *et al.*, 1996; Keel and Defago, 1997; Whipps, 1997a; Nielson *et al.*, 1998; Kang *et al.*, 1998; Kim *et al.*, 1999; Thrane *et al.*, 1999; Nakayama *et al.*, 1999) (Table 11).

Lipopeptide antibiotic surfactin produced by B. *subtilis* GEB3 inhibited the plant pathogens *Pyricularia oryzae* and *Rhizoctonia solani* (Gao-Xuewen, 2003). Another Lipoprotein antibiotic iturin A produced by *B. subtilis* was found effective in suppressing damping-off of tomato caused by *R. solani* in pot tests (Shoda, 1999). Strain PNA1 of *Pseudomonas* spp. produced two phenazine antibiotics, phenazine-1-carboxylic acid and oxychlororaphin, and *in vitro* protected chickpea and pigeonpea plants from Fusarium wilt disease which is caused by *F. oxysporum* f. sp. *ciceri* and *F. udum*, respectively (Vanamala *et al.*, 2003). Alexandrova *et al.* (2002) found *Bacillus subtilis* BS-F3 to produce peptide antibiotic(s) with a wide spectrum of action against *Erwinia amylovora* from different geographic origin and different hosts. Michereff *et al.* (1994) reported that production of antifungal pholoroglucinols by *P. fluorescens* protected sorghum against *Colletotrichum graminicola* mediated anthracnose. *B. subtilis* is known to suppress fungus colonization through production of antibiotics *in vitro* as well as *in vivo* (Brannen, 1995; Kim *et al.*, 1997a; Leifert *et al.*, 1995). Control of *Rhizoctonia solani*

Table 11. Antibiotics or antibiotics-like effectors produced by *Bacillus subtilis* and *Pseudomonas fluorescens.*

Antibiotics	Organisms	References
Bulbiformin	*B. subtilis*	Brannen, 1995
Iturin A	*B. subtilis*	Asaka and Shoda 1996a
Surfactin	*B. subtilis*	Edwards and Seddon, 1992
Agrocin-84	*B. subtilis*	Kim *et al.*, 1997a
Bacillomycin	*B. subtilis*	Besson and Michel, 1984
Mycosubtilins	*B. subtilis*	Peypoux and Michel, 1976
Fengymycin	*B. subtilis*	Vanillakam and Lowffler, 1986
Mycobacillin	*B. subtilis*	Sengupta *et al.*, 1971
Mycocerein	*B. subtilis*	Wakayama *et al.*, 1984
Pyoluteorin	*P. fluorescens*	Whistler *et al.*, 2000
Phenazine	*P. fluorescens*	Schoonbeck *et al.*, 2002
Indole acetic acid	*P. fluorescens*	Mordukhova *et al.*, 2000
N-butylbenzenesulphonamide	*P. fluorescens*	Kim-Keunki *et al.*, 2000
2, 4-Diacetyl pholoroglucinol	*P. fluorescens*	Mazzola *et al.*, 2002
Siderophores	*P. fluorescens*	Perez *et al.*, 2001
Oomycin A	*P. fluorescens*	Gutterson *et al.*, 1988
Tryptophan sidechain oxidase	*P. fluorescens*	Laville *et al.*, 1991
Alginate, HCN, Pseudomonic acid, Oomycin A, 2-Hydroxy-2, 4, 6-cyclo hepta-triene-1-pseudomonic acid, Ovafluorin, Fluopsin C & F, Sorbistin A1 & B, Salicylic acid,	*P. fluorescens*	Johri *et al.*, 1997
Lipodepsipeptides, Syringotoxins, Syringgomycins	*P. fluorescens*	Woo-Sheri *et al.*, 2002

damping-off of tomato by *B. subtilis* RB 14 involved the action of two antibiotics, iturin and surfactin (Asaka and Shoda, 1996a).

Induced systemic resistance

Perhaps the greatest growth area in biocontrol during the last few years has been concerned with induced resistance defined as the process of active resistance dependent on the host plant's physical or chemical barriers activated by biotic and abiotic agents (inducing agents) (Kloepper *et al.*, 1992a). Most work has focused on the systemic resistance induced by non-pathogenic rhizosphere colonizing *Bacillus* and *Pseudomonas* species in systems where the inducing bacteria and the challenging pathogen remained spatially separate for the duration of the experiment, and no direct interaction between the bacteria and pathogen was possible (Sticher *et al.*, 1997; van Loon, 1997). Such split root or spatial root inoculation experiments were used to demonstrate the phenomenon in radish (*Raphanus sativus*) and *Arabidopsis* against *F. oxysporum* (Leeman *et al.*, 1996a; van Wees *et al.*, 1997) and in cucumber (*Cucumis sativus*) against *Pythium aphanidermatum* (Chen *et al.*, 1998). Various combinations of timing and position have indicated that induced resistance also occurs in carnation (van Peer *et al.*, 1991), tobacco (Maurhaufer *et al.*, 1994) and tomato (Duijff *et al.*, 1997). Bacteria differ in ability to induce resistance, with some being active on some plant species and not others; variation in inducibility also exists within plant species (van Loon, 1997). The full range of inducible moieties produced by bacteria is probably not yet known, but lipopolysaccharides (Leeman *et al.*, 1995a) and siderophores (Metraux *et al.*, 1990; Leeman *et al.*, 1996b) are clearly indicated. Van Peer and Schippers (1989) demonstrated that lipopolysaccharides (LPS) extracted from the outer membrane of *P. fluorescens* WCS417 induced systemic resistance in carnation against Fusarium wilt. Following changes may take place in plant roots exhibiting induced systemic resistance as a result of inoculation with biocontrol bacteria:

i. Strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including cellulose, lignin and phenolics (Benhaumou *et al.*, 1996a, b, c, 2000; Duijff *et al.*, 1997; Jetiyanon *et al.*, 1997; M' Piga *et al.*, 1997).

ii. Increased levels of enzymes such as chitinase, peroxidase, polyphenol oxidase and phenylalaline ammonia lyase (M'Piga *et al.*, 1997; Chen *et al.*, 2000).

iii. Enhanced production of phytoalexins (van Peer *et al.*, 1991; Ongena *et al.*, 1999).

iv. Enhanced expression of stress related genes (Timmusk and Wagner, 1999).

However, all these changes generally do not occur in one bacterial-plant combination (Steijl *et al.*, 1999). Similarly, the ability of the bacteria to colonize the internal tissue of the roots has been considered to be an important feature in many of the bacterial root interactions involving induced systemic resistance, but is not a constant feature (Steijl *et al.*, 1999).

Competition for iron

Although competition between bacteria and fungal plant pathogens for space or nutrients has been known to exist as a biocontrol mechanism for many years (Whipps, 1997), the greatest interest recently has involved in competition for iron. Under iron limiting conditions, bacteria produce a range of iron chelating compounds or siderophores which have a very high affinity for ferric iron. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth (O' Sullivan and O'Gara, 1992; Loper and Henkels, 1999). It has been clearly shown that the iron nutrition of the plant influences the rhizosphere microbial community (Yang and Crowley, 2000). Iron competition in pseudomonads has been intensively studied and the role of the pyoverdine siderophore produced by many *Pseudomonas* species has been clearly demonstrated in the control of *Pythium* and *Fusarium* species, either by comparing the effects of purified pyoverdine with synthetic iron chelators through the use of pyoverdine minus mutants (Loper and Buyer, 1991; Duijff *et al.*, 1993). Pseudomonads also produce two other siderophores, pyochelin and its precursor salicylic acid and pyochelin is thought to contribute to the protection of tomato plants from *Pythium* by *Pseudomonas aeruginosa* 7NSK2 (Buysens *et al.*, 1996).

B. subtilis not only produced antibiotics which suppress plant pathogens but also siderophores and the regulation of these products by the gene lpa-14 indicate the possibility of enhanced effectiveness of biocontrol by the manipulation of the

gene (Shoda, 2000). 2,3- Dihydroxybenzoylglycine (2,3-DHBG) is known as a siderophore produced by the gram positive *B. subtilis* (Leong, 1986; Ito and Neilands, 1958). The strains of *B. subtilis* which showed a wide suppressive spectrum on plant pathogens by producing antibiotics, also produce 2,3-DHBG.

Plant growth promotion

Bacillus subtilis and *P. fluorescens* possess great potential to promote plant growth. Their plant growth promoting activity results from the contribution of different components acting either directly of indirectly on the plant. Indirect plant growth promotion is due to suppression of soil-borne plant parasites and deleterious rhizosphere microorganisms, whereas direct plant stimulation is mainly exerted by release of growth factors. These microorganisms may enhance the plant growth through the following mechanisms:

Siderophore synthesis

Strains of *B. subtilis* and *P. fluorescens* can synthesize siderophores that can solubilize and sequester iron form the soil and provide it to plant cells (Glick, 1995). Coinoculation of chickpea with siderophore producing *Pseudomonas* strain CRP55b resulted in the formation of 68-115% more nodules at 80 and 100 days after planting. Khan *et al.* (2002) isolated nine strains of fluorescent Pseudomonads from the rhizospheres of wheat and chickpea and were characterized morphologically as well as biochemically for siderophores activity. All of the strains produced siderophores and were effective in the growth of chickpea. Powell *et al.* (1980) demonstrated the role of iron in crop development and reported the presence of siderophores, particularly hydroxamate siderophore (10^{-7} to 10^{-8} M), in 67 different soils of United States, at concentrations high enough to be useful to plant roots. Akers (1983) also detected siderophore (schizokinen) in paddy crop, suggesting that insoluble form of iron present in the soil, was made available to plants, to a large extent by microbial siderophores. Reid *et al.* (1984) and Barker *et al.* (1985) provided evidences that plants have an ability to incorporate Fe^{3+} of siderophores into their biomass. Sharma and Johri (2003) reported that fluorescent *Pseudomonas* strain GRP3A produced siderophores under iron limited conditions and increased the growth of mungbean. Johri *et al.* (1997) also reported that fluorescent pseudomonad strain

RBT 13 produced siderophores which exhibited *in vitro* antagonism against several bacterial and fungal pathogens and simultaneously increased the growth of four crops.

Phytohormone production

Several strains of *Bacillus subtilis* and *Pseudomonas fluorescens* synthesize phytohormones such as indole acetic acid, gibberellins, cytokinins and zeatin which promote plant growth at various stages (Gracia de Salamone *et al.*, 2001). Plant growth was promoted by *P. fluorescens* by producing cytokinin (Gracia de Salamone *et al.*, 2001), vitamins (Marek-Kazaczok and Skorupsks, 2001) and IAA (Pal *et al.*, 2001). Inoculation with the bacterium enhanced growth in tea, pigeonpea, chickpea and maize (Kumar *et al.*, 1997). Indole acetic acid, gibberellin and zeatin (growth regulators) have been detected from medium cultured with *P. fluorescens* (Meng *et al.*, 1998). Application of the culture solution promoted wheat growth in a manner similar to application of exogenous growth regulators. IAA produced by *P. putida* strain GR12-2 was found to play a major role in the root growth (Patten and Glick, 2002). They primary root system of canola seedlings from seeds treated with IAA producing *P. putida* GR12-2 were on an average 35-50% longer than the roots from the uninoculated seeds. In addition, exposing mungbean cuttings to high levels of IAA by soaking them in a suspension of this bacterial strain stimulated the formation of many, very small adventitious roots.

Bacillus subtilis has also been found as a predominant bacteria in the rhizosphere of some crops (Kloepper *et al.*, 1992b). It has been reported by Archipora *et al.* (2005) *Bacillus subtilis* produces cytokinins which influence the growth of the lettuce plant. Araujo *et al.*, (2005) reported phytohormone production by *B. subtilis* which influences soybean root development. Yuen *et al.* (1985) found that *B. subtilis* strain improved the growth of many plant species in steamed and natural soils. Seed treatment with *B. subtilis* increased the yield of carrots by 48%, oats by 33% and peanuts upto 37% (Weller, 1988). *B. subtilis* B2g caused significant increases of plant growth of cabbage, cucumber and sunflower (Marten *et al.*, 1999). Growth promoting effects of *B. subtilis* and *P. fluorescens* have also been reported on tomato and chickpea crops. *B. subtilis* increases the availability of nitrogen and phosphorus in soil and its application in soil can improve the yield of tomato (Khan and Tarannum, 1999).

Mineral solubilization and synthesis of other compounds

Phosphorus is one of the major plant nutrients which plays a key role in balanced nutrition of plants and thereby in crop production. About 98% of the Indian soils have inadequate supply of available phosphorus and only 0.1% of the total P present in soil is available to plants (Gaur, 1987). To avoid this deficiency, phosphatic fertilizers are added in soil. But most of the water soluble P in the fertilizers becomes insoluble tricalcium phosphate or Fe or Al phosphate complexes and hence unavailable to the growing crops (Gaur, 1990). Hence, solubilization of fixed soil P through the use of microorganisms is a viable option to augment the availability of P in easily assimilable form by crops (Dubey *et al.*, 2000). A number of species of *Bacillus* (Gaur and Gaind, 1987; Kole and Hajra, 1997) and *Pseudomonas* (Dave and Patel, 1999; Reddy and Swami, 2000) are efficient phosphate solubilizers. Application of *Pseudomonas* species increased the P uptake in chickpea by 27% (Alagawadi and Gaur, 1988a), sorghum by 18% (Alagawadi and Gaur, 1988b), potato by 26% (Kundu and Gaur, 1980) and rice by 15% (Gaur, 1990) and increased the yield of crops significantly. Yield of wheat was also increased when *P. straita* was applied along with super phosphate and rock phosphate (Gaur *et al.,* 1980). Gaur and Ostwal (1972) reported that application of *B. polymyxa* in the presence of rock phosphate significantly increased the P uptake of wheat and grain and straw yield. In pot experiments where mustard was grown at different rates of Mussoorie rock phosphate with or without *P. straita,* the microorganism solubilized rock phosphate more efficiently (Dubey *et al.*, 2000). Reddy and Swamy (2000) conducted field experiments on blackgram by applying phosphate solubilizing bacteria, farm yard manure and phosphorus and found improved solubilization of inorganic phosphate compounds and increase in seed yields. Phosphate solubilizing microorganisms produce certain organic acids which are considered the most important mechanism of phosphorus solubilization (Illmer and Schinners, 1995; Yadav and Dadarwal, 1997). Three strains of *P. straita* have been found to produce seven acids such as malic acid, glyoxalic acid, succinic acid, fumaric acid, citric acid, tartaric acid and ketoglutaric acid (Gaur, 1990). A commercial formulation, Microphos based on *B. subtilis* has been developed at IARI which is being successfully used by farmers (Gaur and Gaind, 1984). Both *Bacillus subtilis* and *Pseudomonas fluorescens* have a mechanisms for the solubilization of minerals such as phosphorus that become more readily available for plant growth (Pal *et al.,* 2001). They may also synthesize

some less characterized low molecular mass compounds, enzymes or vitamins that can modulate plant growth and development (Marek-kazaczuk and Skorupska, 2001).

***Bacillus subtilis* against pathogenic fungi**

In vitro fungal growth of *F. oxysporum* f. sp. *ciceri* was effectively inhibited by *B. subtilis* E (Dikkar *et al.*, 2003). Similarly, *B. subtilis* inhibited mycelial growth and sclerotia production of dry root rot of chickpea caused by *R. solani* (Sindhan *et al.*, 2002). Rangeshwaran *et al.* (2002) reported that *B. subtilis* (PDBCEN 3) was antagonistic to *F. oxysporum* f. sp. *ciceri* and provided (38%) inhibition in a dual culture test. Two isolates of *B. subtilis* were found antagonistic to *F. oxysporum* f. sp. *ciceri in vitro* (Dhedhi *et al.*, 1990). Seed bacterization with *Bacillus* spp. reduced the number of wilted chickpea plants in wilt sick plots (Kumar, 1996). Bacterization of chickpea seeds with a siderophore producing fluorescent pseudomonad RBT 13 reduced the number of wilted plants in sick soil by 52% (Kumar and Dubey, 1992).

Podile and Laxmi (1998) reported that cell free culture filtrate of *B. subtilis* AF1 showed a concentration-dependent growth and conidiation inhibition of *F. udum*. An inhibition zone of 3.5 cm against *F. udum* was observed by Goudar *et al.* (2000) *in vitro* studies. Four isolates of *B. subtilis* were found antagonistic to *Botrytis cinerea* in dual culture test (Utkhede *et al.*, 2001). *B. subtilis* produced a wide zone of inhibition against *F. udum* and inhibited spore germination completely at 8×10^7 cells/ml (Sumitha and Gaikwad, 1995).

Lipopeptide antibiotic surfactin produced by *B. subtilis* GEB3 inhibited the plant pathogens *Pyricularia oryzae* and *R. solani* (Gao-Xuewen, 2003). Another Lipoprotein antibiotic iturin A produced by *B. subtilis* was found effective in suppressing damping-off of tomato caused by *R. solani* in pot tests (Shoda, 1999). *B. subtilis* RB 14, produces antibiotics iturin and surfactin which were attributed for suppression of *R. solani* damping-off of tomato (Asaka and Shoda, 1996a). *B. subtilis* reduced root-rot of lentil (*Fusarium avenaceum*) (Hwang, 1994), northern leaf blight of corn (*Exserohilum turcicum*) (Reis *et al.*, 1994), rust infection of safflower (*Puccinia carthami*) (Tosi and Zazzerini, 1994), wilt of cotton (*F. oxysporum* f. sp. *vasinfectum*) (Zhang Jin Xu *et al.*, 1995), Yam leaf spot disease (*Curvularia eragrostridis*) (Michereff *et al.*, 1994) and anthracnose fruit rot of chilli (*Colletotrichum capsici* and *C. gleosporiodes*) (Sariah, 1995). Seed bacterization of pigeonpea with *B. subtilis* AF1 significantly reduced the incidence

of pigeonpea wilt and showed an increase in phenylaniline ammonia-lyase (PAL) and peroxidase activity that are responsible for host plant resistance against the *F. udum* (Podile and Laxmi, 1998). *B. subtilis* reduced the number of cabbage plants damaged by *F. oxysporum* after a direct application of spore suspension to the planting site. A soil treatment with water dispersible granules or spore suspension and a seed treatment resulted in increased emergence rates in cabbage plants inoculated with *R. solani* and *F. oxysporum* (Marten *et al.,* 1999). Seed treatment of pigeonpea with *B. subtilis* and *Trichoderma* spp. effectively controlled pigeonpea wilt and enhanced the yield considerably (Nakkeeran and Renukadevi, 1997).

The mechanism of the action of two strains of *B. subtilis* B4 and B6 against *Gibberella zeae* was based on the production of antibiotic metabolites. The metabolites inhibited the germination of the spores of the pathogen and growth of the mycelia. Salting out with ammonium sulfate and SDS-PAGE showed that antifungal proteins with a molecular weight ranging from 20 to 63 KD were predominant antagonistic substances (Ye *et al.*, 2003). Root rot disease of cauliflower caused by *P. ultimum* var. *ultimum* was significantly controlled by mixing *B. subtilis* in soil rather than by dipping the cauliflower roots in the bacterial suspension (Abdelzaher, 2003). *Bacillus subtilis* strains (BACT-0, BACT-10, AGS-4 and AGS-K) were antagonist towards *B. cinerea* in dual culture (Utkheda *et al.*, 2001). B-916 strain of *B. subtilis* controlled rice sheath blight (*R. solani*) by more than 75% (Tan and Mew, 2001).

B. subtilis CE1 at 10^8 and 10^7 CFU ml^{-1} inocula was able to reduce rhizoplane and endorhizosphere colonization of *Fusarium verticillioides* in greenhouse trials. The strain *B. subtilis* CE1 could be a potential biological control agent against *Fusarium verticillioides* at the root level (Cavaglieri *et al.*, 2005; 2004). *B. subtilis* increased the survival of chickpea/lentil plants and was found effective against root rot/wilt diseases of lentil under greenhouse conditions (Abou-Zeid *et al.*, 2002). A treatment with *B. subtilis* GB03 was found most effective in suppressing fusarial wilt and increasing seed yield (Landa *et al.*, 2004). The interaction study by slide culture technique showed that isolates of *B. subtilis* exhibited bulb formation at hyphal tip of the test fungus and bursted. Soil treatment of *B. subtilis* amended with crustacean chitin significantly ($P \leq 0.05$) reduced infection of *R. solani* and *Fusarium solani* on sunflower and *R. solani* on chickpea. (Sultana *et al.*, 2000). *B. subtilis* GB03 was most effective in reducing stem canker severity

(40-49% reduction) relative to the infested controls over all trials (Brewer and Larkin, 2005). *B. subtilis* was used successfully to control *Pythium* and *Rhizoctonia* diseases of ornamental plants grown in soil treated by aerated steams (Broadbent *et al.*, 1977). In pot soil, infestated with pathogen *R. solani*, diseased incidence was 85% while the introduction of *B. subtilis* into the soil reduced 24% incidence (Asaka and Shoda, 1996a). Khan and Akram (2000) reported satisfactory control of wilt of tomato due to soil application of *B. subtilis*. Study made by Khan and Khan (2001) also demonstrated the potential of another strain of *B. subtilis* in the management of wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici*.

Pseudomonas fluorescens

Several strains of *P. fluorescens* have shown to suppress fungal pathogens *in vitro* and *in vivo*. Seeds treated with *P. fluorescens* strains when sown, the antagonist moved to the rhizosphere and survives there well. Biopriming of seeds increased rhizosphere population. A siderophore producing *Pseudomonas* strain showed *in vitro* antibiotic activity towards 6 species of *Fusarium,* including *F. oxysporum* f. sp. *lycopersici, F. oxysporum* f. sp. *ciceri, F. udum, F. moniliforme, F. solani, F. semitectum* etc. *In vitro* antibiosis of six *P. fluorescens* isolates (GR1, GR25, GR27, WR49, WR55 and WR62) collected from chickpea and wheat rhizosphere resulted in reduction of mycelial growth of *R. solani* and the inhibition zone ranged from 1.3-22.5 mm in different isolates on King's B medium. In a greenhouse study there was a 17.7-100% reduction in rice sheath blight infection due to pretreatment of sclerotia for 4 weeks with suspension of *P. fluorescens* isolates (Pande and Chaube, 2003). Inam ul Haq *et al.* (2003) have found that *P. fluorescens* no. 1, 2, 4, 5 and 9 were effective against wilt fungus reducing incidence by 92, 94, 94, 96 and 96% respectively over control. A plant growth promoting isolate of *P. fluorescens* EM85 strongly antagonized *R. solani,* a causal agent of damping-off of cotton. The isolate produced HCN, siderophore, fluorescent pigments and antifungal antibiotics (Pal *et al.*, 2000). Another isolate, *P. fluorescens* producing pyrollnitrin suppressed root rot of sesamum caused by *Macrophomina phaseolina*. In dual culture test, pyrollnitrin inhibited the mycelial growth of *M. phaseolina* by producing an inhibition zone of 12 mm (Karunanithi *et al.*, 2000). Goudar and Kulkarni (2000) observed an inhibition zone of 7.5 mm that was produced by *P. fluorescens* against *F. udum* in dual culture test. *P. fluorescens* isolated from green gram inhibited the

growth of *Aspergillus* sp., *Curvularia* sp., *F. oxysporum* and *R. solani* in culture. Coinoculation of green gram with this *Pseudomonas* strain S 24 significantly increased the nodule weight, plant dry weight and total plant nitrogen as compared to inoculation with *Bradyrhizobium* alone (Sindhu *et al.*, 1999). *P. fluorescens* was also found to suppress three chickpea pathogenic fungi viz., *F. oxysporum* f. sp. *ciceri, R. solani* and *Pythium* sp. (Nautiyal, 1997). In a green house test, seed dressing of chickpea with *P. fluorescens* increased the germination of seedlings by 25%, and reduced the number of diseased plants by 45% compared to non bacterized controls.

Nautiyal (1997) reported that seed bacterization with *P. fluorescens* strain NBRI 1303 increased the shoot length, dry weight and grain yield, averaging 12%, 18% and 23%, respectively, above untreated controls in field trials. A strain of *P. fluorescens* that produced diacetylphloroglucinol considerably checked the take-all disease caused by *Gaeumannomyces graminis* var. *tritici* under field condition (Marchand *et al.*, 2000). Root rot of cowpea (*R. solani*) was controlled by the application of *P. fluorescens* under field conditions (Barbosa *et al.*, 1995). Soil application of *P. fluorescens* (Khan and Khan, 2001) or its root-dip treatment (Khan and Khan, 2002) satisfactorily controlled tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* and increased the growth and yield of the plants. Talc based formulations of *P. fluorescens* (PDBCAB2) and *P. putida* (PDBCAAB19) were evaluated against natural incidences of wilt and wet root-rot of chickpea. *P. fluorescens* treated plots exhibited lowest root-rot incidence (5%) compared to the pathogen control (14%) and highest plant stand and seed yield. *P. fluorescens* along with *P. putida* suppressed the chickpea wilt by 7.5% (Rangeshwaran *et al.*, 2001). Bacterization of chickpea seeds with a siderophore producing fluorescent pseudomonad reduced the number of chickpea wilted plants in wilt sick soil by 52% (Kumar and Dubey, 1992). *P. fluorescens* 89-61 is a root colonist which has been shown to reduce the disease incidence of *Fusarium* wilt of cotton (Chen *et al.*, 1995), cucumber anthracnose (*Colletotrichum orbiculare*), and bacterial angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*) (Wei *et al.*, 1996). *P. fluorescens* WCS374 controlled the *Fusarium* wilt of radish (Leeman *et al.*, 1995a). Hill *et al.* (1994) reported antagonistic effect of pyrrolnitrin producing *P. fluorescens* (B/L 915) on *R. solani*-inducing damping-off of cotton. De Souza *et al.* (2003) reported that *Pseudomonas* spp. that produces 2, 4 diacetylphloroglucinol caused disorganization of the hyphal tips of *Pythium ultimum* var. *sporangiforum.* Seed

treatment with *P. fluorescens* I10 resulted in the lowest incidence (17%) of pigeonpea wilt (Gholve and Kurundkar, 2002). Selected isolates of *P. fluorescens* and chemical resistance inducers (salicyclic acid, acetylsalicyclic acid, DL-norvaline, indole-3- carbinol and lichenan) were examined for growth promotion and induced systemic resistance against Fusarium wilt of chickpea (cv. JG-62). The isolates (Pf1-94, Pf4-92, Pf12-94, Pf151-94 and Pf179-94) of *P. fluorescens* systematically induced resistance in chickpea *F. oxysporum* f. sp. *ciceri* (FocRs1) and significantly ($P \leq 0.05$) reduced the wilt disease by 26-50% compared to the control (Saikia *et al.*, 2003). Inam ul Haq *et al.* (2001) evaluated different strains of *P. fluorescens* for the biological control of chickpea wilt and found isolates 12 + 15, 11 + 12 + 15, 15 + 11, 15 + 19 and 15 + 14 were the best combination for the biocontrol of *F. oxysporum* f. sp. *ciceri* in water culture as they recorded 98.00, 97, 97, 92 and 92% decrease in disease incidence over the control, respectively. *Pseudomonas fluorescens* strain LRB3W1 inhibited the fungal growth of *Fusarium oxysporum* f. sp. *conglutinans in vitro* and suppressed the fungus under glasshouse conditions (Someya, 2007). The strain LRB3W1 was also found effective in controlling *Fusarium oxysporum* f. sp. *lycopersici* under glasshouse conditions (Someya *et al.*, 2006).

Application of biocontrol bacteria against phytonematodes

Bacillus subtilis

Sikora (1988) reported that the application of *Bacillus subtilis* in sugar beet reduced the infection of *M. incognita, M. arenaria* and *Rotylenchulus reniformis*. El-Sherief and Barakat (1995) have isolated *Bacillus, Cornybacterium, Streptomyces* and *Arthobacterium* species from *M. incognita* egg masses and *Heterodera zeae* cysts. Liquid cultures of these bacteria at a concentration of 0.1 to 0.6% were highly toxic to juveniles of *M. incognita, R. reniformis* and *Tylenchulus semipenetrans*. Similar effects of *B. subtilis* were reported by Khan and Tarannum (1999) on root-knot disease of tomato in a field study. An other field trial has shown suppressive effects of *B. subtilis* on *M. incognita* infecting tomato (Khan and Akram, 2000). Effects of seed treatment (Khan and Kounsar, 2000) and soil application (Khan *et al.*, 2002) with *B. subtilis* were investigated on root-knot of green gram. The treatment decreased the galling by 33% and increased the green gram yield by 22%. Recently, it has been found that application of *B. subtilis* can also control root-knot problem

of seasonal ornamental plants (Khan *et al.*, 2005a). Treatment with *B. megaterium* resulted in a greater than 40% reduction in nematode penetration and decreased the migration of *M. graminicola* to the root zone of rice by 60% compared with that of non treated roots (Padgham and Sikora, 2007). Giannakou *et al.* (2007) evaluated *B. firmus* against root knot nematode in greenhouse and field experiments. A decrease of second stage juveniles hatching from eggs was recorded by using the bio-nematicide at 0.9 g/kg of soil, while further decrease was recorded by doubling the dose. Similarly, *B. firmus* offers a satisfactory and environmentally friendly solution for the control of root-knot nematodes (Ioannis *et al.*, 2004). Jonathan and Umamaheshwari (2006) conducted pot culture study to assess the biocontrol potential of *B. subtilis* isolates (EPB5, 22, 31 and EPC 16) against the root-knot nematode (*Meloidogyne incognita*) of banana. Significant increase in plant growth coupled with reduction in nematode population was observed in the combined treatment of EPB5 + 31.

Pseudomonas fluorescens

Jawaroski *et al.* (1986) showed that two isolates of *P. fluorescens* reduced galling caused by root-knot nematode when inoculated around plant roots while Oostendrop and Sikora (1989) found that 8 isolates of bacteria with three of them being identified as *P. fluorescens* from rhizosphere of sugarbeet suppressed early root infection by *Heterodera schachtii* when applied to seed in non-sterilized field soil in the green house. In the field, all 8 isolates reduced nematode penetration with some reducing nematode number in roots by as much as 75%. Root-dip treatment of rice seedlings with *P. fluorescens* effectively reduced the plant damage and population of *Hirschmanniella gracilis* (Seenivasan and Devrajan, 2000). Amendment of culture filtrate or methanol extract of the culture filtrate of a *T. harzianum* strain Th 6 to *P. fluorescens* growth medium enhanced the production of nematicidal compound(s) by bacterial inoculants *in vitro*. Application of both these microbes in unsterilized sandy loam soil caused greater reduction in *M. javanica* population densities in tomato roots (Siddiqui and Shaukat, 2004). *P. fluorescens* strain CHAO caused a significant reduction in penetration rates of *M. javanica* juveniles in mungbean roots (Siddiqui and Shaukat, 2003). Siddiqui *et al.* (2003) reported that the culture filtrates of *P. fluorescens* strain CHAO caused significant mortality (46%) of *M. incognita* juveniles *in vitro* compared to control (10%). Anita and Rajendran (2002) reported

a significant reduction in nematode population, number of egg masses and gall indices in tomato and brinjal plants in nursery plots where talc based formulation of *P. fluorescens* with CFU 7 x 10^8/g was applied @10 g/m^2 of plot. Siddiqui and Ehteshamul-Haque (2001) reported that *P. aeruginosa* (7.4 x 10^8 CFU/ml) caused the greatest reduction in gall formation due to *M. javanica* (500 J_2/plant). Khan and Mustafa (2005) significantly controlled root-knot of gladioli with root dip treatment with *P. fluorescens*. Soil application of *P. fluorescens* @ 2.5 kg/ha increased plant growth and reduced root-knot population (Senthamarai *et al.*, 2006).

Compatability with chemicals

Although biocontrol agent application is free from health and environmental risks but its action is slow and is required to be applied in advance. Biocontrol agents are also sensitive to soil contamination and their efficacy is affected in the presence of certain toxicants or pesticides. However, all pesticides are not toxic and some biocontrol agents have shown compatibility with pesticides at doses even higher than recommended for soil application. When inconsistency and low efficiency of biocontrol occur in the field, one possible solution is the use of an integrated mixture of chemicals and biological agents. This integration shall be based on a dose of pesticide sufficiently low enough not to cause environmental contamination and other adverse effects. *Trichoderma* strains are naturally resistant to several herbicides, fungicides and pesticides such as DDT and phenolic compounds (Chet *et al.*, 1997). This character represents boader use of *Trichoderma* biopesticides with pesticides. Mukhopadhayay *et al.* (1992) reported that seed treatment with *T. virens* (10^7conidia/ml) along with carboxin (0.1%) was highly effective in managing several soil borne plant pathogens like *Sclerotium rolfsii, Rhizoctonia solani* and *F. oxysporum* in chickpea, lentil and groundnut. Kaur and Mukhopadhayay (1992) reported that a formulation of *T. harzianum* based on wheat bran sawdust showed high potentiality in controlling chickpea wilt complex caused by *R. solani, S. rolfsii* and *F. oxysporum* f. sp. *ciceri*. Integration of *T. harzianum* with a combination of Vitavax-200 + Bavistin and Vitavax-200 + Ziram provided a disease control of 56-63%. *Trichoderma* alone showed 35% disease control, which increased to 46% when integrated with vitavax-200 (seed treatment). Soil drench treatments with captaf and *Trichoderma harzianum* formulation in combination significantly reduced the

root rot mortality of soybean seedlings caused by *Fusarium oxysporum* as compared to control (Kashif *et al.*, 2006). Effective control of bunch rot of grapes was observed by Harman *et al.* (1996) by the integration of *Trichoderma harzianum* with iprodione. Excellent results of integrated control have been attained with strains of *T. virens* and metalaxyl against *Pythium ultimum* infecting cotton (Chet *et al.*, 1997). They also found a combination of *T. virens* and thiram effective to control *R. solani*, infection on tobacco. Seed treatment with *T. virens* (0.5 g) and vitavax 1 g per kg seed gave 51% control of wilt complex in chickpea (Gurha *et al.*, 2003). The integration of *T. harzianum* (10^6 spores/ml/10 g seed) and carboxin (2 g/kg seed) for seed treatment enhanced seed germination by 12-14% and yield by 43-73% and reduced the wilt incidence by 44-60% (Dubey *et al.*, 2007).

The *Trichoderma* formulation efficiently controlled several phytopathogens such as *R. solani*, *P. ultimum* or *Sclerotium rolfsii* when alternated with methyl bromide, benomyl, captan or other chemicals (Vyas and Vyas, 1995). Treatment of soil in containers with methyl bromide at a dose equivalent to a commercial application of 500 kg/ha did not reduce the soil population of naturally occurring *Trichoderma* spp. and allowed rapid colonization by the introduced strain of *T. harzianum* (Chet, 1990).

Chickpea seeds treated with powdered preparations of *T.* (= *Gliocladium*) *virens* in combination with vitavax (carboxin) showed colonization of *T. virens* on seed coat (testa), collar region, plumule and radicle. The highest population was observed in the spermosphere (7×10^5/g) followed by the rhizosphere (6.3×10^4/g), when seeds treated with *T. virens* + vitavax were sown in pathogen *F. oxysporum* f. sp. *ciceri* infested soil (Tewari *et al.*, 2003). Excellent control of *Pythium ultimum* have been achieved with strains of *T. virens* and metalaxyl in cotton (Chet *et al.*, 1997), *Verticillium dahliae* by *T. harzianum* and captan in potato, and *Rhizoctonia solani* by *T. virens* and thiram in tobacco and other crops (Chet *et al.*, 1997). Application of *T. harzianum* T-35 along with benomyl was more effective to check the *Verticillium* wilt of potato under field conditions (Chet, 1990). Similarly, *T. harzianum* applied immediately after soil fumigation with methyl bromide (500 kg/ha), followed by planting of peanuts, blight and root rot, caused by *S. rolfsii* and *R. solani,* respectively, were controlled better than where methyl bromide was used

alone. Soil fumigation controlled the diseases but the soil was rapidly reinfested by the pathogens. *T. harzianum* prevented or delayed reinfestation of the soil in the fumigated field. The combination of fumigation and *T. harzianum* killed almost all sclerotia of these pathogens in field. Transplanting peanut plants treated with *T. harzianum* into soil fumigated with methyl bromide resulted in significantly less diseases caused by *S. rolfsii* and *R. solani* and increased the yield of peanuts (Elad *et al.*, 1982).

B. subtilis with added chemicals yielded more effective control of onion white rot caused by *Sclerotium cepivorum* (Utkhede and Rahe, 1981) or root rot of field pea caused by *R. solani* (Hwang and Chakravarty, 1992) than chemicals alone, and the bacteria yielded season long protection. Application of *B. subtilis* RB14-C in conjugation with chemical pesticide flutolanil effectively control the damping-off in tomato plants caused by *Rhizoctonia solani*. The synergistic phenomenon involved in integrated application of fungicides and biocontrol agents may be more efficient and longer lasting than the control achieved through biocontrol agents or fungicides alone (Shoda, 2000).

The efficacy of *Pseudomonas fluorescens* strains CP 8-2 and CP 8-3 was tested in combination with thiram against collar rot (*Sclerotium rolfsii*) of chickpea cv. Annigeri both in greenhouse and field. Greenhouse studies indicated that the combination treatment of CP 8-3 with thiram was superior in minimizing collar rot incidence (57.6 and 64.8%) in both the seasons compared to the control. In the field experiments, the combination treatments also proved superior in recording low disease incidence which ranged from 7.3 to 15.5% in Annigeri compared to 25.2% in the control (Singh *et al.*, 2003).

Biopesticides

It would be unrealistic to expect that biological control agents can completely replace chemical pesticides in the crop protection. There are, however, areas in which biological agents are superior to chemical agents, and thus future research needs to be directed towards exploiting such niches. For any crop protection agent, an efficient formulation is a necessity to translate laboratory activity into adequate field performances. It is encouraging that many companies world over are engaged with programmes to develop commercial products or biopesticides of various beneficial organisms (Table 12).

Biopesticides are advantageous due to their ecosafety, target specificity, non development of resistance that so often renders pesticides obsolete and ineffective at reduced number of applications giving higher yield with improved quality of the produce. A huge amount of food material worth of more than Rs. 4000 crores is rejected annually because of very high pesticide residue contents (Singhal, 2004). Biopesticide application is absolutely free from residual accumulation, and have great acceptability. In fact, biopesticides are important component of organic farming.

The global share of biopesticides in agrochemical market in 2000 AD amounted to around 10% with a growth rate of 10-15% per annum compared to 2% for chemical pesticides. In India, the present consumption levels of biopesticides has increased from around 1% share in 2001 to around 2.5% in 2003 and is expected to reach 12-15% by the end of 2005 (Singhal and Sharma, 2003). Although some decrease in pesticide consumption has appeared or the use of biopesticide is on the increase, but not to the desired level of growth. Various small entrepreneurs are coming up with the biopesticides, but many of them have little or no quality consciousness. Several reviews (Cate, 1990; Hemming and Houghton, 1993; Wilson and Wisniewski, 1994) have addressed aspects of maximizing chances of developing a successful biopesticide that are summarized as following. The likelihood for successful development of a commercially viable biopesticide is optimized once all these criteria are fulfilled.

i. Effective suppression of the fungal pathogen before it causes economically important disease to crop.
ii. Consistant performance under authentic conditions of crop management and the crop environment.
iii. Adaptation to existing integrated pest management (IPM) schemes of disease control.
iv. Price competitiveness with other means of combating the same target pest.
v. Compatability with other chemical or biological treatments targetting other pest(s).
vi. Adaptation to commonly used farm agronomic methodologies.
vii. Preservation of naturally occurring beneficial antagonist(s) of related or non-related pests.
viii. User and environment friendliness.

Table 12. Some important biopesticides being currently formulated in India and abroad.

Products	Biocontrol agents	Name of the manufacturer
Pant Biocontrol	*Trichoderma harzianum*	Deptt. of Plant Pathology, G.B.P.U., Pantnagar
Agent 1 and 3	*Pseudomonas fluorescens*	
Biowilt-X	*T. harzianum*	Deptt. of Plant Protection, AMU, Aligarh
Bionem-X	*Pochonia chlamydosporia*	Deptt. of Plant Protection, AMU, Aligarh
Biocomp-X	*P. fluorescens*	Deptt. of Plant Protection, AMU, Aligarh
Biocure-X	*B. subtilis*	Deptt. of Plant Protection, AMU, Aligarh
Actiguard	Acibenzolar-S-methyl	Syngenta Crop Protection, Greensboro
Actinovate	*Streptomyces lydicus*	Natural Industries, Houston, USA
AQ10	*Ampelomyces quisqualis*	Ecogen, Inc. Israel
Anti-Fungus	*Trichoderma* spp.	Grondortsmettingen De Cuester, Belgium
Aspire		*Candida oleophola* Ecogen, Inc. Israel
Bactophyte	*Bacillus subtilis*	Russia
Biofungus	*Trichoderma* spp.	Grondortsmettingen De Cuester n.v., Belgium
Bas-derma	*T. viride*	Basarass Biocontrol Res.Lab., India
Binab-T	*T. harzianum, T. polysporum*	Bio-Innovation AB, UK
Bioderma	*T. viride/T. harzianum*	Biotech International Ltd., India
Biofox C	*Fusarium oxysporum*	S .I. A. P. A., Italy
Bioject Spot-Less	*Pseudomonas aureofaciens*	Eco Soil Systems, USA
Bio-Save 10LP	*P. syringae*	CTT Corp., USA
Bio-Save 11	*P. syringae*	EcoScience Corp., USA
Blight Ban A506	*P. fluorescens*	Plant Health Technologies, USA
Blue Circle	*Burkholderia cepacia*	EcoScience Corp., USA
Cedomon	*P. chloraphis*	Bio Agri AB, Sweden
Coniothyrin	*Coniotyrium minitans*	Russia
Companion	*Bacillus subtilis* GB03	Growth Products, USA
	B. lichniformis, B. megaterium	
Contans, Intercept	*Coniothyrium minitans*	Prophyta Biolog. Pflanzenschutz, Germany
Conquer	*P. fluorescens*	Mauri Foods, Australia and Sylvan Lab, USA
Deny	*B. cepacia, P. cepacia*	Stine Microbial Products, Shwanee, KS
DiTera	*Myrothecium verrucaria*	Valent Biosciences, USA
Ecofit	*T. viride*	Hoechst Schering Afgro Evo Ltd., India
Epic	*Bacillus subtilis*	Gustafson Inc., USA
Fusaclean	*F. oxysporum*	Natural Plant Protection, France
Kalisena	*Aspergillus niger*	Cadilla Pharma., India
Galtrol-A	*Agrobacterium radiobacter*-84	AgBioChem Inc., USA
Intercept	*P. cepacia, B. cepacia*	Soil Technologies Corp., USA
HiStick N/T	*B. subtilis*	MicroBio Group, UK
Kodiak, Kodiak	*B. subtilis*	Gustaffson Inc., USA
Koni	*Coniothyrium minitans*	BIOVED Ltd., Hungary
Messenger	*Erwinia amylovora* HrpN	BIOVED Ltd., Hungary
Mycostop	*Streptomyces griseoviridis*	Kemira Agro. Oy, finland
Nogall, Diegall	*Agrobacterium tumefaciens*	Bio-Care Technology Pvt. Ltd., Australia
Norbac 84 C	*A. radiobacter* strain 84	New BioProducts Inc., USA
Paecil	*Paecilomyces lilacinus*	Tech. Innov. Corp. Pvt. Ltd., Australia
Phagus	Bacteriophage	Natural Plant Protection, France
Polyversum	*Pythium oligandrum*	Biopreparatory Ltd., Czech Republic
PSSOL	*P. solanacearum*	Natural Plant Protection, France
Funginil	*T. viride*	Crop Health Bioproduct Res. Centre, India
Ecoderma	*T. viride* + *T. harzianum*	Margo Biocontrol Pvt. Ltd. Banglore
Defence	*T. viride*	Wockhardt Life Science Ltd. Mumbai
Trichoguard	*T. viride*	Anu Biotech Int. Ltd. Faridabad

Formulations and substrates

Media for immobilization

Microbial products for agriculture must be sold in such a form that can easily be used by the farmers. They must be supplied to farmer in a familiar format that does not require new equipment, technology, or application technique. Microbial product must also be formulated to preserve cell viability and maximum biological activity under prolonged conditions of transits and storage. In India many industries are engaged in developing commercial formulations of biocontrol agents for the management of different plant pathogens. It is important that a biocontrol agent needs to be incorporated in a suitable carrier medium for its storage in a viable state and for field application at a later stage. Various types of crop residues, agricultural wastes, and a number of inert materials such as talc powder, vermiculite, perilite, sand etc. have been used as carrier media (Kousalya and Jeyarajan, 1990; Mukhopadhyay, 1987; Bhai *et al.,* 1994). Tiwari *et al.* (2004) evaluated grains of sorghum, wheat, bajra, wheat bran, rice bran and sugarcane bagasse for mass multiplication of *T. viride.* Grains of sorghum (cv. swanki) were found the best substrate that provided maximum spore concentration (8 x 10^9 spores) and spore viability (92.5%) after 15 days of incubation at 27± 1°C. Spores remained viable for 6 months at 5°C. Wheat bran and sawdust mixture have been used as a carrier media for the mass multiplication of *T. harzianum* (Elad *et al.,* 1980; Mukhopadhyay *et al.,* 1986). Khan *et al.* (2001) evaluated various agricultural and industrial wastes for mass multiplication of *T. harzianum, T. virens* and *P. chlamydosporia,* and found highest CFU, 1.2x10^6 on bagasse-soil-molasses for *T. harzianum*, 1.0x10^6 for *T. virens* and 1.1x10^6 on corn meal-sucrose mixture for *P. chlamydosporia.* Vidhyasekaran *et al.* (1997) developed powder formulations of *P. fluorescens* using talc powder, peat, vermiculite, lignite and kaolinite. Bhai *et al.* (1994) evaluated a number of agricultural wastes which could be used as carrier and multiplication media at the same time. They reported that sterilized tea waste, coffee husk or a mixture of coffee husk and cattle manure were ideal combinations for the fast growth and multiplication of *T. harzianum* and *T. virens.* Angappan (1992) used molasses yeast medium for growing *T. viride* and mixed it with talc powder to develop commercial formulation. The initial population in the produce was 3 x 10^8 CFU/g, whereas the product should contain 2 x 10^7 CFU/g at the time of use. The shelf life of this product was 4 months. Seed treatment of chickpea with this product

maintained the rhizosphere population of the bioagent at 11-13 x 10^3 CFU/g soil throughout crop. Ranganathan *et al.* (1995) found that gypsum is a good and cheap substitute for talc. Nakkeeran and Jeyarajan (1996) tested two industrial wastes, precipitated silica and calcium silicate as carrier for *Trichoderma* in the place of talc. The material gave a population of 0.99 and 1.04 x 10^8 CFU/g, respectively compared to 1.4 x 10^8 CFU/g in talc substrate after 4 months of storage. Both the substrates were much cheaper than talc. Backman and Rodriguez-Kabana (1975) used diatomaceous earth granules impregnated with 10% molasses solution for rearing *T. harzianum.* It was applied to peanut at 140 kg/ha on 70 and 100 days after sowing to control *Sclerotium rolfsii.* The disease was reduced by 42% over control and yield increased by 13.5%.

Several researchers have used combination of two or more agricultural materials. Elad *et al.* (1986) used wheat bran : saw dust : tap water mixture (3:1:4 v/v) for *T. harzianum.* It was applied at the time of sowing and mixed with the soil to a depth of 7-10 cm with a rotatory hoe. It increased yield of beans (15 q/ha), tomato (3 q/ha), cotton (5 q/ha) and potato (4-6 q/ha) and controlled *Sclerotium rolfsii* and *Rhizoctonia solani.* Vidhya (1995) applied the formulation of *T. harzianum* based on vermiculate - wheat bran (@ 250 kg/ha) to mung bean and found 41% reduction in root-rot (*Macrophomina phaseolina*) and 91% increase in yield. Papavizas and Lewis (1989) prepared *T. virens* on alginate-bran-fermenter biomass pellets and pyrax-fermenter biomass mixture. Soil application of the product checked the damping-off caused by *R. solani.* Several other substrates such as farm yard manure (FYM), biogas plant slurry, press mud, paddy chaff, rice bran, groundnut shell (Kousalya and Jeyarajan, 1988), FYM, FYM + Sand, Saw dust, Wheat bran, pigeonpea leaves, wheat straw, and urd bean straw (Chaudhary and Prajapati, 2004) have been tested to grow *T. viride* and *T. harzianum.* The enumeration of viable CFUs revealed that pigeonpea leaves and urd bean straw were the best substrates showing 3.4 and 3.4 x 10^5 propagules at 4 months, 1.2 and 1.1 x 10^5 at 8 months and 1.5 and 3.0 x 10^4 at 12 months of storage at room temperature, while sorghum seed showed 11.4, 3.8 and 0.6 x 10^4 propagules at the same intervals, respectively. Next suitable substrates were wheat straw and sawdust. Cabanillas and Barker (1989) tested some carriers like wheat grains, alginate pellets, and diatomaceous earth granules for soil application of *P. lilacinus.* Kerry *et al.* (1984) used oat seeds to rear *P. chlamydosporia* for field application. Soil application of the colonized oat kernels

@ 0.5 and 1.0% (w/w soil : seed) considerably reduced the population of root-knot and cyst nematodes (Godoy *et al.*, 1983; Rodriquez - kabana *et al.*, 1984). De Leij and Kerry (1991) did encapsulation of liquid suspension of spores and hyphae of *P. chlamydosporia* with sodium alginate containing 10% (w/v) kaolin or wheat bran. On soil application, the fungus proliferated in soil from only those granules which contained wheat bran as energy source. In other study, Kerry (1988) estimated approximately 9 x 10^4 and 4 x 10^4 CFUs of the fungus/g soil after 1 and 12 weeks of application of granules, respectively.

Development of biopesticides

A commercial formulation of a biocontrol agent (biopesticide) can be developed following the methodology described below (Whipps, 1992).

Isolation and screening of antagonist

The process starts with the isolation of an efficient strain of an antagonist with regard to disease suppression, fast multiplication, competent and surviving ability. Thereafter *in vitro* screening of the antagonist is done for the desired characters. It is quite inexpensive and indicates the mode of action, easy to culture, and survival test in environmental extremes within which the antagonist have to act. A large number of isolates can be tested in a short time to select the most suitable one.

Pot test

Performance of the biocontrol agent should be tested first under pot condition (controlled conditions) to examine its effectiveness against the target pathogen (s) infecting the plant.

Field trial

Promising antagonists from pot tests should be used for field trials to ascertain their efficacy under field conditions. Since variation in weather influences disease incidence, trials should be conducted for at least 15-20 sites-years (sites x years). This is, however, costly and labour intensive.

Toxicological data

It includes information on antagonists, safety to man, plants, animals and survival of other antagonists in cohabitance in the field.

Commercial data

It includes cost of production, easy to use and relative efficacy with fungicides or cultural methods. If they are ecofriendly, slightly less efficacy or increased cost may be acceptable. It must fit with integrated control.

Scale ups

This involves methods of inoculum production, formulation, quality standards, shelf life and development of a suitable delivery system to treat soil, seed, or plant parts during growth. The cropping system should be taken into account. Other disease control tactics like soil or seed treatments, fungicidal spray, which may interfere with the activity of biocontrol agent, should be considered. Strains suitable for each soil condition should be selected. The effect of control of target pathogen on other pathogens should also be studied.

Fermentation methods

Fermentation methods are important for mass production of microorganisms and to harvest a much better yield quantitatively as well as qualitatively. Three methods of fermentation are described by Lewis *et al.* (1991).

Liquid fermentation

This technology has been adopted to produce bacterial and fungal biomass. A suitable medium should consist of inexpensive, readily available agricultural byproducts with appropriate nutrient balance. Acceptable materials include molasses, brewer's yeast, corn steep liquor, sulphate waste liquor, cotton seed and soya flours (Lisansky, 1985). A higher produce of *Trichoderma* chlamydospores was harvested through liquid fermentation technology. The preparation based on chlamydospores prevented the disease more effectively than a preparation that contained conidia only (Lewis *et al.*, 1990; Papavizas and Lewis, 1989). Small scale fermentation in molasses-brewers yeast medium has also resulted in abundant chlamydospore production of *Trichoderma* (Papavizas *et al.*, 1984).

Solid fermentation

Mass production of antagonists on solid substrates for the production of inoculum of various biocontrol fungi includes straws, wheat bran, saw dust; bagasse moistened with water or nutrient solutions through fermentation technology is referred to as solid fermentation (Papavizas, 1985). This technology is also effective especially for those organisms which can multiply on dry substrates.

Semisolid fermentation

Semisolid fermentation is done for the fungi which do not sporulate in liquid culture. Diatomaceous earth granules impregnated with molasses (Backman and Rodriquez-kabana, 1975), wheat bran and vermiculite-wheat bran (Lewis *et al.,* 1989) yield good produce of bioagents. This method, however, requires more area, labour intensive and the chances of contamination are high when compared to liquid fermentation.

Registration

Current legislation demands that new products are subjected to detailed study of their environment impact and toxicological effects and they are registered. As current legislation stands, there are certain categories of biocontrol agents that have an easier and quicker passage for registration. Indigenous microorganisms that are specific to a defined group of targets have a comparatively straightforward progress. Under the Section 9(3) of Pesticide Act of India (1968) information required for registration of any biopesticides are: scientific name and common name; natural occurrence and morphological descriptions; details of manufacturing process (active and inert ingredients of formulation); Test methods (dual culture of pathogenicity); Quantitative analysis (cfu on selective medium, absence of Gram negative bacteria contaminants); moisture content; shelf life; mammalian toxicity; bioefficacy. environmental toxicity and residue analysis. Because of less awareness of growers towards biocontrol programmes, the Indian Biopesticide Industry involves more than 15-20% expenditure on marketing compared to only 1-2% marketing expenses in the case of conventional pesticides (Singhal and Sharma, 2003). So there is a need for simplification of registration requirements and government subsidies should be granted to farmers to promote biopesticide use.

The registration policies may vary with the country. In USA, registration of microbial pesticide requires toxicological tests for oral, dermal, eye and other health hazards using test animals or fish. If these tests show no adverse effects and the biocontrol agent is not a pathogen, it is registered and can be sold. The cost required for research and development for biopesticide is only US$ 0.8-1.6 million as against US$ 20 millions for chemical pesticides. The toxicological tests for a biocontrol agent cost US$ $0.5x10^6$ as against US$ $10x10^6$ for chemical pesticides. The number of candidates to be tested to develop one biocontrol product will be in 100s as against 20, 000 for a chemical pesticide. It was estimated that the market size required for profit for a biocontrol agent is US$ 1.6 million per year as against US$ 4 million per year for a chemical pesticide (Cook, 1993).

Quality control

Quality control is the most essential aspect of biopesticide production. A good quality of the preparation is necessarily required to retain the confidence of farmers on the efficacy of biocontrol formulation. Being living agents their population in a product may be influenced by storage. The other contaminating microorganisms in the product should also be within permissible limits.

Shelf life

The biggest obstruction in commercialization of a biocontrol agents preparation is its loss of viability of the biocontrol agents over time. Considerable efforts have been made in India itself to determine the viability of biocontrol agents in their preparations when stored at room temperature and in refrigerator. Most of the results are variable and therefore it appears that shelf life is also dependent upon species/ isolate/strain.

In general the antagonist multiplied in an organic food base has greater shelf life than that on an inert or inorganic food base (Jeyarajan and Nakkeeran, 1996). Talc based formulations of *T. harzianum* can retained more than 10^6 viable propagules per g upto 90 days (Prasad and Rangeshwaran, 2000). A talc based preparation of *T. virens* conidia retained 82% viability at 5°C in refrigerator after 6 months, while at room temperature (25-35°C) same level of viability was observed only up to 3 months. Lewis *et al.* (1995) reported that among the different carriers tested; the shelf life of *B. subtilis* in soybean flour was increased upto three months. Storage

at 5°C increased the shelf life of *T. virens* and *T. hamatum* in granular formulations of pre gelatinizing starch flour upto 6 months. Chlamydospores based formulations of *T. virens* and *T. harzianum* exhibited longer shelf life (80% viability for 7 months) than conidia based formulations (80% viability after 4 months) at room temperature (Mishra *et al.*, 2001). Jeyarajan *et al.* (1994) developed talc, peat, lignite and kaolin based formulations of *T. viride,* which had a shelf-life of 4 months. Ranganathan *et al.* (1995) also reported 4 month shelf life of *T. viride* in gypsum based formulations. Studies on storage temperature revealed that 20-30°C was optimum to store vermiculite fermentor biomass of *Trichoderma* upto 75 days without loosing the viability (Nakkeeran *et al.*, 1997).

Methods of application of biopesticides

It is rather necessary to have an efficient, economic and ecologically viable mode of application of biocontrol agents in soil ecosystem. To achieve effective biocontrol, formulated products must be delivered effectively into appropriate agricultural system. It should suit the available equipments like seed drill, seed treatment drum, sprayers etc. It should be easy to perform and should be placed in the site of action (soil, seed, wound, foliage etc.). For this purpose several methods have been developed.

Seed treatment

Seed coating with biocontrol agents has emerged as a feasible way of delivering the antagonist for the management of plant diseases. Delivery of antagonists to the spermosphere is the most effective and economical method. For effective lodging of bioagent, the seed is first coated with some sticky material such as gum arabica, sucrose or molasses. Thereafter, a formulation is applied to the seeds and mixed thoroughly to achieve homogenous distribution of the biocontrol agent. Wells *et al.* (1972) was probably the first to demonstrate the efficacy of *T. harzianum* against *Rhizoctonia* spp. through seed treatment. Khan *et al.* (2004, 2005a) found a significant control of root-knot and wilt of chickpea through seed treatment with *T. harzianum, P. chlamydosporia* or *P. fluorescens* formulations.

Seed pelleting with *T. harzianum* using 5 x 10^9 CFU/ml reduced the infection of *M. phaseolina* in bean and chickpea (Jeyarajan and Ramakrishnan, 1991).

Jeyarajan *et al.* (1994) developed talc based formulation of *T. viride* for seed treatment which reduced the root rot incidence by 60 (urdbean), 50 (chickpea), 77 (peanut) and 67% sesamum and increased the yield by 20, 13, 12 and 12%, respectively. Seed treatment with *T. harzianum* or *T. viride,* significantly reduced root rot incidence to 10 and 13%, respectively, compared to 60% incidence in the control (Sankar and Jeyarajan, 1996). Seed treatment of pigeonpea with *Trichoderma* spp. and *Bacillus subtilis,* effectively controlled root-rot and wilt of pigeonpea and enhanced the yield considerably (Nakkeeran and Renukadevi, 1997; Nakkeeran *et al.,* 1995).

Seed bio priming

Seed bio priming is a process that involves treating of seeds with biocontrol agents followed by incubation under warm and moist conditions for a duration just prior to radical emergence. This technique has potential advantages over simple coating of seeds as it results in rapid and uniform seedling emergence as well as uniform colonization by the biocontrol agent on the seed surface. Vidhyasekaran and Muthamilan (1995) showed that when chickpea seeds were given bio priming treatment with *Pseudomonas fluorescens*, the population of biocontrol agent was significantly higher in the rhizosphere than those without bio priming. Population of *T. harzianum* and *T. virens* on the surface of treated seeds of tomato, brinjal, soybean and chickpea increased from @ 10^4 CFU/seed before bio priming to 10^6CFU/seed after bio priming (Mishra *et al.*, 2001). In an infested field with *Pythium ultimum,* the plant stand increased to 70-80% as against 10% in control due to priming of *Trichoderma* spp. Tomato seeds have shown ten fold increase in the population of *Trichoderma* following solid matrix priming with the antagonist (Harman *et al.,* 1989).

Soil application

There are several reports on the application of biocontrol agents to the soil and other growing media either before or at the time of planting for control of a wide range of soil-borne fungal pathogens (Baby and Manibhushanrao, 1993, 1996). Soil application of *T. harzianum, P. chlamydosporia* and *P. fluorescens* @ 50 kg formulation/ha effectively controlled the chickpea and pigeonpea wilt, root-knot and disease complex caused by *Fusarium* and *Meloidogyne* spp. concomitantly

(Khan, 2005). Adams (1990) defined efficiency of biocontrol agents as the ratio of number of propagules of mycoparasites required to obtain disease control to the typical inoculum density of a plant pathogen. For controlling *R. solani,* 5 x 10^6 CFUs of *Trichoderma* was required for each propagule of *R. solani.* Addition of wheat bran based inoculum to soil gave 80% reduction of root-rot over control in chickpea and bean (Elad *et al.,* 1986). Incidence of urdbean root-rot was reduced by 91% by adding *T. viride* + *T. harzianum* to soil (Kousalya and Jeyarajan, 1988). Delivering *T. harzianum* through soil during sowing increased the percentage of survival of peanut (90%), while in control none of the plants survived (Muthamilan and Jeyarajan, 1996). In pot experiments conducted in a glass house, augmentation of the soil with *T. harzianum* in maize meal-sand medium @ 40-60g/ kg soil resulted upto 89% reduction of wilt disease of pigeonpea over control (Biswas and Das, 1999).

Foliar spray

Existence of epiphytic microflora on plant surface including leaves and flowers is a natural phenomenon. There are many studies where phyllosphere microflora has been implicated in reduction of foliar diseases (Chattopadhyay and Nandy, 1982; Bora *et al.*, 1993; Haware *et al.*, 1996). Antagonists isolated from phyllosphere can be introduced on the host surface by spraying their propagules. Bacteria are better colonizer than fungi in this respect (Parashar *et al.*, 1992). Application of *B. subtilis* to bean leaves found in decreasing incidence of bean rust, *Uromyces phaseoli* as effectively (75%) as was the weekly treatments with mancozeb (Baker *et al.,* 1983). *Pseudomonas* applied to beet leaves actively competed for amino acids on the leaf surface and inhibited spore germination of *Botrytis cinerea, Cladosporium herbarium* and *Phoma betae* (Blakeman and Brodie, 1977). Four applications of a conidial suspension of *Ampelomyces quisqualis* (12 x 10^4 conidia/ml) at weekly intervals were applied to cucumber leaves. The treatment suppressed conidial production and viability of powdery mildew (Jayarajan and Nakkeeran, 1996).

MATERIALS AND METHODS

Isolation and identification of the wilt fungus

The root and stem samples of chickpea plants showing characteristic wilt symptoms were collected from naturally infected farmer's fields in Aligarh to isolate wilt fungus *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. f. sp. *ciceri* (Padwick) Synd. & Hans. The samples were placed in plastic bags during transport to the laboratory. The samples were cut into small pieces (2-5mm) and were surface sterilized in 2.5% (vol/vol) NaOCl for 15s, rinsed once in sterile distilled water and blotted dry on a sterile paper towel. The pieces were placed aseptically to a Petri plate containing solidified potato dextrose agar (PDA) (Appendix 1) with 5% (vol/vol) lactic acid to inhibit bacterial growth. The inoculated plates were incubated in an incubator at 25±2°C. The fungal colonies developed in the plates were sub cultured on PDA slants. The fungi developed on slants were examined microscopically to identify and isolate pure culture of *F. oxysporum* f. sp. *ciceri*. Temporary slides of the fungus were prepared in cotton blue and was identified on the basis of its cultural and morphological characters (Jalali and Chand, 1992). The fungus was also compared with the standard culture procured from the IARI, New Delhi and IMTECH, Chandigarh. The isolate got identified from the NBAIM, Mau, and RRL-CSIR, Jammu, India where it has been deposited.

Mass culture of the wilt fungus and pathogenicity test

Pure cultures were established for pathogenicity testing by transferring stored cultures to plated PDA and incubated at 25±2°C for 5 days. The local isolate of *F. oxysporum* f. sp. *ciceri* was multiplied on sorghum seeds, which were soaked overnight in 5% sucrose and 0.0003% chloramphenicol solution (Whitehead, 1957). The seeds were transferred to conical flasks of 500 ml capacity. The flasks were autoclaved twice at 15kg/cm^2 pressure at 121°C for 15-20 minutes. Thereafter, the flasks were inoculated with the pure culture of *F. oxysporum* f. sp. *ciceri* and incubated for 8-10 days in an incubator at 25±2°C. During incubation, the flasks were shaken manually for a few minutes daily for uniform colonization of seeds. The inoculum so prepared was incorporated in the pots containing sterilized soil (2 g/kg soil) and mixed thoroughly. Five replicates were maintained and in each pot

surface sterilized seeds (five) of chickpea were sown. The pots were irrigated with tap water regularly to maintain adequate moisture. Symptoms developed were observed 30 days after sowing. To fulfill Koch's postulates, the pathogen was reisolated from roots and/or stem of infected plants. Inoculation and pathogenicity tests were done as described above.

Isolation, identification and mass culture of root-knot nematode, *Meloidogyne incognita*

Infected root samples of eggplant, *Solanum melongena* L. showing root-knot symptoms were collected from fields. The samples were brought to lab and association of *Meloidogyne incognita* was confirmed using perineal pattern technique (Barker *et al.*, 1985). Pure culture of *M. incognita* was prepared by single egg mass inoculation technique (Khan and Khan, 1991). A female along with the attached egg mass was excised from a gall. The species, *M. incognita* was identified on perineal pattern characters. Thereafter, the egg mass was placed near the roots of a seedling of eggplant, cv. Pusa Purple Long grown in sterilized soil in a clay pot. The nematode culture from this plant was raised in sterilized soil on eggplant in numerous pots. For field inoculation, the nematode culture was prepared from the egg masses excised from pot grown eggplants. The egg masses were placed on wire gauze in a Bearmann funnel and incubated at 25-30°C for 6-10 days. The hatched juveniles were collected from the funnel.

Biocontrol agents

Biocontrol fungi

Standard cultures of fungal antagonists viz., *Trichoderma harzianum* Rifai, *T. virens* (J. H. Miller, Giddens & A. A. Foster) Arx *and Pochonia chlamydosporia* (=*Verticillium chlamydosporium*) (Goddard) Zare and W. Gams were obtained from MTCC, Chandigarh. Fungal antagonists (five isolates) were collected from rhizosphere of healthy chickpea plants adjacent to or between two wilted plants and were identified on the basis of cultural and morphological characters. *Trichoderma* specific medium (TSM) (Elad *et al.*,1983) (Appendix 2) and a semi-selective Medium (Appendix 3) (De Leij and Kerry, 1991) were used for isolation of *Trichoderma* species and *P. chlamydosporia* from soil. Soil was processed using standard serial dilution technique (Waksman, 1927) and 0.3 ml of 10^{-4} dilutions was

plated on respective sterilized and solidified media in Petri plates. The plates were incubated at 25±2°C for 5-10 days for the growth of the fungi. The isolates were also got identified from the RRL, Jammu, India and NBAIM, Mau, India, where they have been deposited. The isolated cultures were labeled as (Table 13).

Table 13. Standard and local isolates of the biocontrol fungi used in the study.

T. harzianum	*T. virens*	*P. chlamydosporia*
Th 00*	Tv 00*	Pc 00*
Th 01	Tv 01	Pc 01
Th 02	Tv 02	Pc 02
Th 03	Tv 03	Pc 03
Th 04	Tv 04	Pc 04
Th 05	Tv 05	Pc 05

* Standard strains

Screening of biocontrol fungi against the wilt fungus

The fungal antagonists were screened against the wilt fungus, *F. oxysporum* f. sp. *ciceri in-vitro* for the following tests.

Monoculture growth rates of isolates of biocontrol fungi and wilt fungus

Strains/isolates of *T. harzianum, T. virens, P. chlamydosporia* and wilt fungus *F. oxysporum* f. sp. *ciceri* were cut (six mm PDA discs) from five days old culture plates with a sterilized cork borer. The discs were placed individually at the centre in 9 cm Petri plates containing solidified PDA. The plates were incubated at 25±2°C for 7 days and radial growths were measured.

Dual culture test

The strains/isolates of biocontrol fungi were evaluated against *Fusarium oxysporum* f. sp. *ciceri* in laboratory by dual culture technique (Morton and Stroube, 1955). Six mm PDA discs of *F. oxysporum* f. sp. *ciceri* and *Trichoderma* spp. were cut from the five days old culture plates with sterilized cork borer and placed on solidified PDA 5 cm apart in the central area in Petri plates. The plates were incubated at 25±2°C. The process was repeated for nine consecutive days. The observations on the growth and ability of biocontrol agents to restrict and colonize *F. oxysporum* f. sp. *ciceri* were recorded on 10th day of inoculation of the first set.

Mycoparasitism

From the zone of interaction in dual culture plates, the mycelial mats were picked gently by a sterilized needle and put in a drop of cotton blue stain on a microscopic slide. The mycelial mats were then transferred to a clean slide in a drop of lactophenol. The mycelium was teased by a needle, covered with a cover slip and observed under a compound microscope at 10x, 40x and 100x magnifications to determine penetration and lysis of the pathogenic mycelium by *Trichoderma* spp.

Antibiosis

Antibiosis is one of the most important attribute in deciding the competitive saprophytic ability of *Trichodema* spp. Antibiosis occurs when toxic metabolites or antibiotics either volatile or non-volatile produced by one organism have a direct effect on another organism. Effect of volatile and non-volatile compounds produced by the *Trichoderma* spp. on *Fusarium oxysporum* f. sp. *ciceri* was studied in Petri plates.

Volatile compounds

PDA discs (6 mm) of five days old culture of strains/isolates of *Trichoderma* spp. were placed aseptically on solidified PDA at the centre in Petri plates (Dennis and Webster, 1971a). The plates were incubated at 25±2°C for 2, 4, 8 and 10 days followed by immediate replacement of lid by a solidified PDA freshly inoculated with *F. oxysporum* f. sp. *ciceri.* A set of plates with PDA medium without the biocontrol agents at the lower side and *F. oxysporum* f. sp. *ciceri* inoculated plates at the upper side served as control. The plate in pair were sealed together with cellophane adhesive tape and incubated for 10 days at 25±2°C. Three plates were maintained for each treatment. After incubation, the colony diameter of *F. oxysporum* f. sp. *ciceri* in different plates was measured and growth inhibition was calculated according the formula:

$$I = C - T/C \times 100$$

Where, I = Percent growth inhibition; C = Radial growth in control (mm); T = Radial growth in treated plates (mm).

Non-volatile compounds

Conical flasks containing 150 ml potato dextrose broth were inoculated with 6 mm discs of 5 days old culture of isolates and strains *Trichoderma* spp. and *Pochonia chlamydosporia* separately, and were placed on an electric shaker inside an incubator for 15-25 days. Thereafter the broth was filtered through Whatman No. 42 filter paper under aseptic conditions and the filtrate was collected in sterilized Erlenmeyer flasks. The culture filtrates thus obtained, was centrifuged at 6000 rpm for 15 minutes to make it cell free.

Antifungal effect

The cell free filtrate of *Trichoderma* spp. was added to melted double strength PDA (40°C) to obtain final concentration of 10%, 25% and 50% (vol/vol); 20 ml of this medium was poured into sterilized Petri plates. After solidification, the plates were centrally seeded with 6 mm discs of the *F. oxysporum* f. sp. *ciceri* from 5 days old PDA culture and were then incubated in an incubator at 25±2°C for 10 days. A set of plates inoculated with the test pathogen not amended with the culture filtrate served as a check. Three replicates for each treatment were maintained. Observations on radial growth of mycelium were recorded periodically (Dennis and Webster, 1971b) and percent inhibition of colonization by *F. oxysporum* f. sp. *ciceri* was determined by the formula:

$$I = C - T/C \times 100$$

Where, I = Percent growth inhibition; C = Radial growth in control (mm); T = Radial growth in treated plates (mm).

Antinematode effect

The cell free culture filtrates of the biocontrol agents were also tested for antinematode effect on hatching and mortality of *Meloidogyne incognita*. Five ml filtrate was transferred to a glass cavity block to which 10 surface sterilized egg masses of relatively same size were added. The egg masses were surface sterilized by immersing in 0.5% NaOCl for 2-3 minutes. Thereafter, the masses were rinsed several times with distilled water on a sterilized 60-mesh sieve (250 μ) to remove the residues of NaOCl. Two sets of blocks in which egg masses were immersed in distilled water and broth alone (uninoculated) served as control. The cavity blocks were incubated at 25-27 °C for 5 days in an incubator. To avoid evaporation during

the incubation, the blocks were placed inside Petri dishes containing 10 ml distilled water and covered with their lids. Three cavity blocks were maintained for each treatment and the experiment was conducted three times. After incubation, larvae present in the suspension were counted.

To examine the effect on mortality of juveniles, 5 ml culture filtrate was placed in a glass cavity slide to which 1 ml suspension containing approximately 100 freshly hatched and surface sterilized juveniles (J_2) of *M. incognita* was added. The juveniles were surface sterilized by immersing in 0.5% NaOCl for 2-3 minutes and were immediately rinsed several times with distilled water on a sterilized 500 mesh sieve (24 μ) to remove the residues of NaOCl. Juveniles kept in the broth alone (without microorganisms) and in distilled water served as control. Each treatment was replicated three times. The slides were placed on glass supports in Petri dishes containing distilled water and covered with a lid having water soaked blotter paper and incubated at 25-27°C for 4 days. After incubation, numbers of dead juveniles characterized by the straight and immobile body posture were counted. The experiment was repeated three times.

Compatibility of the biocontrol agents with pesticides

Six pesticides viz., carbendazim (Bavistin 50 WP), mancozeb (Dithane M-45 75 WP), metalaxyl (Apron 35 SD), captan (Captaf 50 WP), thiram (TMTD 75 WP) and nemacur (Fenamiphos) were tested against the biocontrol agents using poisoned food technique (Grover and Moore, 1961). Fifty ml aliquots of PDA (double strength) taken in an Erlenmeyer flasks of 250 ml capacity and sterilized in an autoclave. Different concentrations of the pesticides from 10, 25, 50, 125, 250, 500, 1000, 2000, 3000 and 5000 μg/ml were prepared in distilled water. Fifty ml of a concentration was aseptically transferred to the Erlenmeyer flask containing 50 ml PDA. Five Petri plates (90 mm diameter) for each concentration of the fungicides were prepared by pouring 20 ml PDA aliquots in each plate and allowed to solidify. Thereafter the plates were seeded centrally with a 3 mm disc of 4 days old culture of *T. harzianum, T. virens* and *P. chlamydosporia*. PDA plates without a fungicide but inoculated with the fungi served as a control. The inoculated plates were incubated at 25±2°C for 5 days. The radial growth of the colony in each treatment was measured and the percent inhibition of growth was calculated by the formula:

$$I = C - T/C \times 100$$

and ED_{90} (maximum inhibition concentration) and ED_{50} (safe tolerance concentration) were determined.

To determine the compatability of *B. subtilis* and *P. fluorescens* with same fungicides and nematicide, 25 to 50,000 µg/ml concentrations were prepared in double distilled water. Double strength nutrient agar was used as medium for both the bacteria. Twenty ml of nutrient agar containing desired concentration was poured in Petri plates and left over night to observe contamination, if any. Thereafter, 0.1 ml of overnight cultures (10^7 CFU) was spread over the solidified plates with a glass spreader. The plates were incubated at 30±2°C for 24 hrs and bacterial colonies were identified and counted.

Biocontrol bacteria

Standard strains of *Bacillus subtilis* (Ehrenberg) Cohn and *Pseudomonas fluorescens* Migula were procured from MTCC, Chandigarh. Five local isolates of each bacterium were also isolated from different crop fields using the Maintenance Medium for *Bacillus subtilis* (MMBC) (Appendix 4) and King's B supplemented with 45 mg novobiocin, 44.9 mg penicillin and 75 mg cycloheximide/kg + 20 mg tetracycline/ litre for *P. fluorescens* (Appendix 5). The bacteria were characterized biochemically as recommended by the Society of American Bacteriologists and the properties were compared with that of the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The isolates were also identified from RRL, Jammu and NBAIM, Mau, India where they have been deposited. The isolates were labelled as indicated in the Table 14.

Table 14. Standard and local isolates of the biocontrol bacteria used in the study.

Bacillus subtilis	*Pseudomonas fluorescens*
Bs 00*	Pf 00*
Bs 01	Pf 01
Bs 02	Pf 02
Bs 03	Pf 03
Bs 04	Pf 04
Bs 05	Pf 05

* Standard strains

Biochemical characterization of bacterial strains

Fermentation

Carbohydrates such as polysaccharides, disaccharides and monosaccharides serve as main source of energy for microorganisms. Some microorganisms ferment a wide variety of these substances whereas others utilize only a few carbohydrates. Depending upon the species, various end products are formed such as organic acids (lactic, acetic, butyric, propionic); neutral products (acetone, hydrogen and carbondioxide). Formation of acids and gas is detected by using phenol red as indicator, which remains red at neutral pH (7.0) and turns yellow in acidic medium (pH 6.9). Production of gas is detected using a Durham tube.

The bacterial strains/isolates were inoculated in phenol-red nutrient broth (Appendix 6) containing different sugars viz., glucose, fructose, maltose, sucrose and lactose. The broths were incubated for three days at 30±2°C. The observations were recorded for the production of acid if the colour of medium changed from red to yellow or gas seen in Durham tube, shows positive reaction for carbohydrate fermentation.

Ammonification

The organic nitrogen compounds are subject to dissimilation by a wide variety of heterotrophic microorganisms to yield ammonia and other end products. In this experiment, peptone broth, which contains an organic nitrogen substrate, was used to test the ability of strains/isolates of *B. subtilis* and *P. fluorescens* to degrade proteins with the resultant formation of ammonia. After incubation, the presence of ammonia, indicative of ammonification, is detected by the yellow colour when Nessler's reagent is added to samples of test cultures.

Peptone broth (4%) (Appendix 7) taken in culture tubes was autoclaved at 15 kg/cm^2 pressure at 121°C for 15 to 20 minutes. The tubes were inoculated with a loopful of culture of the strains/isolates of *B. subtilis* and *P. fluorescens* and incubated at 28±2°C for 48 hrs. A drop of culture broth was placed on a clean and dry glass slide to which one drop of Nessler's reagent was added, change of colour to yellow is indicative of positive for presence of ammonia.

Antibiotic sensitivity test

Antibiotic sensitivity test of bacterial isolates was performed according to Bauer *et al.* (1966). The strains/isolates of *B. subtilis* and *P. fluorescens* were cultured on nutrient broth in culture tubes to obtain good turbidity (10^9 CFU/ml). The culture was spread on solidified nutrient agar (Appendix 8) (Hi-Media India) in Petri plates (0.1 ml/plate) by a glass spreader. The plates were kept at room temperature for 10 minutes to absorb the extra moisture. Antibiotic discs were then aseptically mounted over the agar surface and the plates were incubated at 35±2°C for 48-72 h. The zones of inhibition were scored for resistance and sensitivity by comparing with the chart diameter given by the disc manufacturer (Hi-Media India).

Catalase test

Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. Inoculum of strains/isolates of *B. subtilis* and *P. fluorescens* were picked from a plate culture and placed on a slide. One drop of hydrogen peroxide was added causing liberation of oxygen as gas bubbles, which indicates the presence of catalase in the culture.

Citrate-utilization test

The citrate test identifies the use of citrate as a sole carbon source, since there are no other nutrients in this medium. The basic end products will cause the brom thymol blue indicator in the medium to turn from forest green to royal blue.

Petri plates having solidified Simmon's citrate agar (Appendix 9) were streaked with strains/isolates of *B. subtilis* and *P. fluorescens* and incubated at 25 or 37° C for 2 days. Change in colour of agar from green to blue was indicative of positive for citrate utilization.

Gelatin hydrolysis

Gelatin is a protein that gets solidified at room temperature. If the bacterium produces the enzyme gelatinase (which optimally is produced at 25°C), the gelatin is hydrolyzed and gets liquified. In this experiment, gelatin medium (Appendix 10) was inoculated with strains/isolates of *B. subtilis* and *P. fluorescens* and incubated at 28±2° C for 48 h. After incubation the tubes were refrigerated at 4°C for 20 minutes and determined for positive test for gelatin hydrolysis on the basis of physical state of the medium.

Indole test

Indole is a nitrogen containing compound formed from the degradation of the amino acid tryptophan. The ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms, and therefore serves as a biochemical marker. Tryptone contains considerable amount of tryptophan that is used to detect indole production by the organism. The presence of indole is detected by adding Kovac's reagent (Appendix 11) which produces a cherry-red reagent layer. The reagent is prepared from p-dimethylaminobenzaldehyde, butanol and hydrochloric acid. Indole is extracted from the medium into the reagent layer by the acidified butanol component and forms a complex with p-dimethylaminobenzaldehyde, yielding the cherry-red colour. Cultures producing a red reagent layer are indole positive whereas, its absence indicates a negative reaction.

Strains/isolates of *B. subtilis* and *P. fluorescens* were inoculated in the nutrient broth in culture tubes and incubated at 30±2°C for 24-48 h. After the incubation, few drops of Kovac's reagent were added to the broth and the formation of the red ring was observed.

Levan formation

Solidified King's medium supplemented with 4% sucrose in Petri plates was inoculated with *B. subtilis* and *P. fluorescens*. The plates were incubated at 28±2°C for 48 h. After incubation slimy colonies with excess levan formation were taken as positive.

Methyl red test

The methyl red test determines the use of glucose, with the subsequent production of acid, tested by the pH indicator methyl red. It turns red at pH range of 4.0 which is an indication for positive test whereas, at a pH 6.0, still indicating the presence of acid but with a lower hydrogen ion concentration, methyl red turns yellow indicating a negative reaction.

The strains/isolates of *B. subtilis* and *P. fluorescens* were inoculated in methyl red Voges Proskuer (MR-VP) broth (Appendix 12) in culture tubes and incubated at 28±2°C for 24-48 h after which the results were observed for change of colour. On incubation if broth remained red, indication for positive test and yellow colour for negative test.

Nitrate reduction test

This test determines the ability of microorganisms to reduce nitrates (NO_3) to nitrites (NO_2) or further to reduce nitrites to ammonia (NH_3+) or molecular nitrogen (N_2). Nitrate reduction can be determined by cultivating organisms in a nitrate broth medium. After incubation of microorganisms in culture broth, reagent A (sulfanilic acid) (Hi-Media India) and reagent B (dimethyl alpha naphthylamine) (Hi-Media India) are added to the broth. If nitrate reduction is carried out by the organism, cherry red colour appears immediately.

The strains/isolates of *B. subtilis* and *P. fluorescens* were inoculated in trypticase nitrate broth (Appendix 13) and incubated at 35±2°C for 48 h. Appearance of red colour on addition of the two reagents was indication for positive reaction.

Oxidase test

This test measures the ability of a microbe to oxidize certain aromatic amines e.g., p-aminodimethyaniline, to form coloured end products. The oxidation correlates to high cytochrome oxidase activity in some bacteria. Discs impregnated with oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) are placed over the bacterial colonies. In a positive test the disc turns deep purple blue whereas no colour change indicates absence of oxidase activity.

Strains/isolates of *B. subtilis* and *P. fluorescens* were streaked on nutrient agar in Petri plates and incubated for 48 h at 28±2°C. Thereafter, oxidase discs from (Hi-Media, India) were placed on the streaks. The discs, if turned white to deep blue within 10-15 seconds, is indicative of positive for oxidase.

Starch hydrolysis

Starch is a complex polysaccharide that cannot be utilized by the bacteria directly for energy. The enzyme amylase is excreted out of the cells (an exoenzyme) into the surrounding media, catalyzing the breakdown of starch into sugars. Starch agar medium is used to demonstrate the hydrolytic activities of these exoenzymes. The detection of the hydrolytic activity following the growth period of bacteria is made by performing the starch test to determine the presence or absence of starch in the medium. Starch in the presence of iodine imparts a blue-black colouration to the medium, indicating the absence of starch-splitting enzymes and representing a

negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis surrounds the microorganism colony, and a positive for starch hydrolysis.

Test organisms were streaked on starch nutrient agar plates and incubated 28±2°C for 2-3 days. The plates were then flooded by Gram's iodine (Hi-Media, India) and observed for zone of clearance, which is indicative for positive response.

Tripple sugar iron agar test (TSI)

The carbohydrate utilization patterns are determined in the TSI agar slants containing 1% each lactose and sucrose and 0.1% glucose. The acid-base indicator phenol red is incorporated to detect carbohydrate fermentation that is indicated by a change in colour of the medium from orange-red to yellow in the presence of acids.

Strains/isolates of *B. subtilis* and *P. fluorescens* were inoculated in the TSI agar slants (Appendix 14) and incubated at 30°C for 18-24 h. After incubation, phenol red indicator was added and colour was observed for fermentation.

Voges-Proskauer Test

The Voges-Proskauer test determines the glucose use to produce nonacidic or neutral end products called acetoin (or acetylmethylcarbinol). Development of a deep rose colour in the culture 15 minutes following the addition of Barritt's reagent is indicative for the presence of acetylmethylcarbinol (positive) where as the absence of rose colouration is a negative result.

Strains/isolates of *B. subtilis* and *P. fluorescens* were inoculated in Methyl Red-Voges Proskauer broth (Hi-Media, India) and incubated for 2-3 days at 28±2°C. After the incubation Barrit's reagent (Appendix 15) was added gradually in drops and the change in colour to red indicates positive test.

Tests for screening antagonism and growth promotion by the bacteria

HCN production

The strains and isolates of *B. subtilis* and *P. fluorescens* were streaked on Petri plates containing sterilized trypticase-soy-agar (Appendix 16) (Bakker and Schipper, 1987). A Whatman filter paper no. 2 soaked in alkaline picric acid solution was placed in the lid of each plate. The plates were incubated at 28±2°C for 4 days and

change in colour of the filter paper from yellow to orange brown was recorded as positive for HCN.

Antifungal activity on solid media

The strains/isolates of *B. subtilis* and *P. fluorescens* inoculated on PDA in Petriplates were first incubated in the dark at 30°C for 24 h. Mycelial discs, cut from actively growing colonies of pathogenic fungus were placed 3 cm apart from the bacterial colonies. The plates were incubated at 28±2°C for 6 days and the percent growth inhibition of *F. oxysporum* f. sp. *ciceri* was calculated by the formula used earlier. A control was maintained with *Fusarium oxysporum* f. sp. *ciceri* inoculated mycelial disc placed on PDA in the plate without bacteria and five replicates were maintained for each treatment.

Effect on nematode hatching and mortality

The bacterial strains were routinely grown overnight at 28±2°C with mild shaking in King's B (*P. fluorescens*) and nutrient broth (*B. subtilis*). The cultures were centrifuged twice at 6000 rpm for 20 minutes. The pellet was discarded and the supernatant was collected in a beaker. To determine the nematicidal activity with regard to hatching of eggs, 5 ml filtrate was transferred to a glass cavity block to which egg masses of similar size surface sterilized (0.5% NaOCl) were added. Two sets of cavity blocks had egg masses in distilled water and broth alone (uninoculated) served as control. The cavity blocks were incubated at 25-27°C for 8 days in an incubator. To avoid evaporation during the incubation, the blocks were placed inside Petri dishes containing 10 ml distilled water and covered with their lids. Three blocks were maintained for each treatment and the experiment was conducted three times. After incubation, the larval suspension were counted under the stereomicroscope.

To examine the effect on mortality of juveniles, 1 ml pure culture filtrate was taken in a glass cavity slide to which 1 ml suspension containing 100 freshly hatched juveniles of *M. incognita* and surface sterilized with 0.5% NaOCl was added. Juveniles kept in the broth alone (without any bacteria) or in distilled water served as control. Each treatment was replicated three times. The slides were placed on glass supports inside Petri dishes containing distilled water and incubated at 25-27°C for 2 days. After incubation, number of dead juveniles were counted. The experiment was performed three times.

Colorimetric quantification of indole acetic acid

Quantification test for IAA was done according the standard method (Gordon and Weber, 1951; Brick *et al.,* 1991). A loopful of test organism was inoculated in 10 ml Luria Bertani broth (Appendix 17) amended with tryptophan 35 mg/100 ml, and was incubated at 28°C for 24 h on a rotary shaker. The broth was centrifuged at 10,000 rpm for 15 minutes, 2 ml of the supernatant was taken to which 2-3 drops of O-phosphoric acid was added. Four ml of $FeCl_3HClO_4$ reagent (Appendix 18) was added to the aliquot. The samples were incubated for 25 minutes at room temperature and percent absorbance was read at 530 nm in a spectrophotometer (Spectronic 20, USA). Concentration of IAA produced by cultures was measured with the help of standard graph of IAA in the range of 10-100μg ml^{-1} (Loper and Schroth, 1986). Similarly, quantitative estimation of IAA was performed at different concentrations of tryptophan (0, 50-500μg ml^{-1}).

Phosphorus solubilization

The test bacterial isolates were screened for their phosphate solubilization activity on Pikovykaya's agar medium (Pikovskaya, 1948) (Appendix 19). Freshly grown bacterial cultures were spot inoculated on Pikoyskaya medium containing tricalcium phosphate (0.5%) and incubated at 28±2°C for 7 days. Colonies of the strains/isolates of *B. subtilis* and *P. fluorescens* showing a clearing (solubilization) zone were considered as phosphate solubilizers.

Quantitative estimation of phosphate solubilization in liquid medium

Test organisms were cultured in 100 ml Pikovskaya's liquid medium for 5 days at 28±2°C. The culture broth was centrifuged at 15, 000 rpm for 30 minutes. Two ml supernatant was taken in a culture tube to which 10 ml chloromolybydic acid (Appendix 20) was added. The mixture was shaken thoroughly and diluted with distilled water to 45 ml. Thereafter 0.3 ml chlorostannous acid (Appendix 21) was added and the final volume was made to 50 ml. After 10 minutes, the resultant blue colour (% transmittance) was measured at 430 nm in a spectrophotometer. Quantification values were recorded from a standard curve prepared with various phosphorus concentrations (Appendix 22).

Screening of industrial/agricultural wastes and materials to rear the biocontrol fungi

To mass culture the biocontrol fungi viz., *T. harzianum*, *T. virens* and *P. chlamydosporia* for application, following industrial/agricultural wastes and materials were screened:

1. Corn cob
2. Corn meal
3. Corn grain
4. Oat kernel
5. Wheat grain
6. Wheat meal
7. Bagasse - soil - molasses mixture (4:1:2)
8. Inert charcoal
9 Corn cob - sand mixture (4:1)
10. Sawdust
11. Husk - sand mixture (4:1)
12. Fly ash
13. Diatomaceous soil
14. Compost
15. Mustard cakes
16. Leaf litter

Media 1-4: The material was soaked overnight in water containing 5% sucrose and chloramphanicol (30 mg/lit).

Media 5-6: The material was soaked for 6 h in water containing 5% sucrose and chloramphanicol (30 mg/lit).

Media 7- 8: The material was sprinkled with 5% molasses solution (0.5 ml/g material).

Media 15 -16: The material was sprinkled with water (0.5 ml/g material).

The material after soaking/sprinkling were filled in conical flasks and autoclaved twice at 15 kg/cm^2 pressure for 15 minutes. Next day, pure culture of the biocontrol agents was inoculated in the conical flasks. The flasks were incubated in a BOD incubator at 25±2°C for a week. During incubation the flasks were shaken periodically to achieve uniform colonization by the fungi.

Mass culture of biocontrol fungi and bacteria

Trichoderma* species and *Pochonia chlamydosporia

Bagasse-soil-molasses (BSM) was used to prepare mass culture of *T. harzianum, T. virens* and *P. chlamydosporia*. The bagasse, soil and molasses were mixed in the ratio of 4:1:2 (5% solution in water) and filled in conical flasks of 500 ml capacity.

The flasks were sealed with cotton plugs and butter paper and autoclaved two times at 15 kg/cm^2 pressure at 121°C for 15-20 minutes. The flasks were inoculated with *T. harzianum, T. virens* and *P. chlamydosporia* separately and incubated in a BOD incubator at 27±2°C for 8-10 days. For large-scale production, BSM medium was filled in heat resistant plastic bags and autoclaved twice at 15 kg/cm^2 pressure at 121°C for 15-20 minutes. The medium in polybags was inoculated with the colonized medium from conical flasks. After inoculation, the bags were sealed and kept in an incubator at 27±2°C for 8-10 days and shaken periodically. During this period the BSM mixture was fully colonized by the antagonistic fungi and ready for field application. Before application, colony forming unit (CFU) count of the biocontrol agents in the BSM was determined using dilution plate method.

Bacillus subtilis* and *Pseudomonas fluorescens

Nutrient broth was prepared in 1000 ml conical flasks and autoclaved at 15 kg/cm^2. After cooling, the flasks were inoculated with a loopful of two days old culture of both the bacteria separately and incubated at 35±2°C for two days to get a good turbidity.

Pot culture experiment to test effectiveness of the biocontrol agents against the target diseases

Pot experiments were conducted to determine the efficacy of biocontrol agents against wilt (*Fusarium oxysporum* f. sp. *ciceri*), root-knot (*Meloidogyne incognita*) and disease complex (*F. oxysp.* f. sp. *ciceri* + *M. incognita*) of chickpea. A total of 672 clay pots (15 cm diameter and height) were filled with steam-sterilized soil amended with compost (3:1), and following 32 treatments each for soil application and seed treatment were incorporated. Six replicates were maintained for all treatments. Additional 9 replicates were taken for nematode treatments which were used to monitor nematode soil population monthly.

01. Plant (Uninoculated Control)
02. Plant + *Trichoderma harzianum*
03. Plant + *T. virens*
04. Plant + *Pochonia chlamydosporia*
05. Plant + *Bacillus subtilis*
06. Plant + *Pseudomonas fluorescens*

07. Plant + Carbendazim
08. Plant + Nemacur
09. Plant + *F. oxysporum* f. sp. *ciceri* (Fungus inoculated control)
10. Plant + *T. harzianum* + *F. oxysporum* f. sp. *ciceri*
11. Plant + *T. virens* + *F. oxysporum* f. sp. *ciceri*
12. Plant + *P. chlamydosporia* + *F. oxysporum* f. sp. *ciceri*
13. Plant + *B. subtilis* + *F. oxysporum* f. sp. *ciceri*
14. Plant + *P. fluorescens* + *F. oxysporum* f. sp. *ciceri*
15. Plant + Carbendazim + *F. oxysporum* f. sp. *ciceri*
16. Plant + Nemacur + *F. oxysporum* f. sp. *ciceri*
17. Plant + *Meloidogyne incognita* (Nematode inoculated control)
18. Plant + *M. incognita* + *T. harzianum*
19. Plant + *M. incognita* + *T. virens*
20. Plant + *M. incognita* + *P. chlamydosporia*
21. Plant + *M. incognita* + *B. subtilis*
22. Plant + *M. incognita* + *P. fluorescens*
23. Plant + *M. incognita* + Carbendazim
24. Plant + *M. incognita* + Nemacur
25. Plant + *Fusarium oxysporum* f. sp. *ciceri* + *M. incognita* (concomitantly inoculated control)
26. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *T. harzianum*
27. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *T. virens*
28. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *P. chlamydosporia*
29. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *B. subtilis*
30. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *P. fluorescens*
31. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + Carbendazim
32. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + Nemacur

Field trial

An experiment was conducted in a field of 50 x 30 m in the Faculty Farm, Aligarh Muslim University, during November, 2004 - March, 2005 and November, 05-March, 06. The land was prepared by adding 4 tonnes farm yard manure during ploughing. The major climatic conditions viz., temperature, humidity and rainfall were 17.8^{0}C (11.0-29.9), 64% (42.0-77.8) and 10 mm (0-63.2 mm)

(during Nov., 04-March, 05) (Appendix 24) and 17.8°C (11.0-25.2), 67.7% (54.9-75.7) and 1.5 mm (0-14.6 mm) (during Nov., 05-March, 06) (Appendix 25) respectively. The soil was sandy clay loam (66.7% sand, 19% silt, clay 14.3%), with 43% water holding capacity, 7.9 pH, 0.016 organic carbon, 1.9% organic carbon and 15.2 kg/ha available phosphorus. In the field 144 microplots each of 2x4 m dimension were prepared with 0.5 m wide and 0.25 m high bunding (margins) to permit flood irrigation to individual plots. The width of the bunding was considered adequate to minimize possible lateral movement of nematodes or microorganisms. The following 24 treatments were maintained for each seed and soil application of biocontrol agents. Same treatments were also used to test efficacy of the biopesticides based on *Trichoderma harzianum, Pochonia chlamydosporia* and *Bacillus subtilis* next year in an adjoining field.

01. Plant (Control)
02. Plant + *T. harzianum*
03. Plant + *P. chlamydosporia*
04. Plant + *B. subtilis*
05. Plant + Carbendazim
06. Plant + Nemacur
07. Plant + *Fusarium oxysporum* f. sp. *ciceri* (Fungus inoculated control)
08. Plant + *T. harzianum* + *F. oxysporum* f. sp. *ciceri*
09. Plant + *P. chlamydosporia* + *F. oxysporum* f. sp. *ciceri*
10. Plant + *B. subtilis* + *F. oxysporum* f. sp. *ciceri*
11. Plant + Carbendazim + *F. oxysporum* f. sp. *ciceri*
12. Plant + Nemacur + *F. oxysporum* f. sp. *ciceri*
13. Plant + *M. incognita* (Nematode inoculated control)
14. Plant + *M. incognita* + *T. harzianum*
15. Plant + *M. incognita* + *P. chlamydosporia*
16. Plant + *M. incognita* + *B. subtilis*
17. Plant + *M. incognita* + Carbendazim
18. Plant + *M. incognita* + Nemacur
19. Plant + *M. incognita* + *Fusarium oxysporum* f. sp. *ciceri* (Concomitantly inoculated control)

20. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *T. harzianum*
21. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *P. chlamydosporia*
22. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + *B. subtilis*
23. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + Carbendazim
24. Plant + *M. incognita* + *F. oxysporum* f. sp. *ciceri* + Nemacur

For each treatment, three microplots (replicates) were maintained which were randomly distributed in the field.

Application of pathogens and biocontrol agents

Fusarium oxysporum f. sp. *ciceri*

Sorghum seeds colonized by *F. oxysporum* f. sp. *ciceri* were grinded with known volume of distilled water in an electric grinder. Ten ml suspension containing 2 g fungus colonized seeds (Table 16) was mixed in 1 kg soil filled in a pot. For field trials, the fungus was applied @ 2 g/kg soil in top layer. Weight of the top soil to 10 cm depth in a microplot of 2x4 m was estimated as 355 kg. Hence, the fungus suspension containing 710 g colonized seeds grinded in 10 litre tap water was sprinkled in a microplot to achieve uniform distribution of the pathogen. The inoculation was done two days prior to seed sowing. The CFU load of *F. oxysporum* f. sp. *ciceri*/g seed is given in Table 14.

Meloidogyne incognita

Nematode inoculation was done @ 2000 second stage juveniles/kg soil in both pots and field trials. Ten litres water containing 7,10,000 second stage juveniles of *M. incognita* was added to a microplot to obtain uniform population of the nematode. The amount of inoculum level was determined considering 355 kg soil/microplot. The inoculation was done a day before the fungus inoculation.

Trichoderma harzianum, T. virens and *Pochonia chlamydosporia*

In pot study, 1 g of biocontrol fungi cultured on baggasse-soil-molasses (BSM) (Table 15) was applied to a pot filled with 1 kg sterilized soil. Whereas, in field trial, soil application of the biocontrol fungi or its biopesticides was done @ 40 g material/microplot at the time of seed sowing. The application was done in rows where the seeds were to be sown. For seed treatment, the dose was 5 g BSM or

biopesticide/kg seed that was applied to seeds along with the commercial rhizobium of chickpea strain ($25x10^8$ CFUs/g formulation).

Table 15. Colony forming units (CFU) load of inocula of fungi and bacteria at the time of application.

	Pot Expt.	Field - Expt. I	Field- Expt. II
Soil Application:			
F. oxy. f. sp. *ciceri*	$21x10^8$ CFUs/g SS	$20x10^8$ CFUs/g SS	$22x10^8$ CFUs/g SS
Rhizobium	$25x10^8$ CFUs/g F	$24x10^8$ CFUs/g F	$26x10^8$ CFUs/g F
T. harzianum	$20x10^9$ CFUs/g BSM	$22x10^9$ CFUs/g BSM	$24x10^9$ CFUs/g BP
T. virens	$15x10^9$ CFUs/g BSM	-	-
P. chlamydosporia	$5x10^8$ CFUs/g BSM	$6x10^8$ CFUs/g BSM	$8x10^8$ CFUs/g BP
B. subtilis	$10x10^{13}$ CFUs/ ml NB	$10x10^{13}$ CFUs/ ml NB	$12x10^{13}$CFUs/g BP
P. fluorescens	$12x10^{14}$ CFUs/ml NB	-	-
Seed Treatment:			
T. harzianum	$8x10^8$ CFUs/seed	$10x10^8$ CFUs/seed	$12x10^8$ CFUs/seed
T. virens	$5x10^8$ CFUs/seed	-	-
P. chlamydosporia	$2x10^7$ CFUs/seed	$3x10^7$ CFUs/seed	$5x10^7$ CFUs/seed
B. subtilis	$8x10^{12}$ CFUs/seed	$7x10^{12}$ CFUs/seed	$9x10^{12}$ CFUs/seed
P. fluorescens	$11x10^{13}$ CFUs/seed	-	-

SS, fungus colonized sorghum seeds; F, formulation; BSM, bagasse-soil-molasses; NB, nutrient broth; BP, biopesticide.

Bacillus subtilis* and *Pseudomonas fluorescens

For pot inoculation 1 ml of two days old pure culture of the biocontrol bacteria in nutrient broths (Table 15) diluted with 10 ml double distilled water was added per pot. For field trials, 40 ml of two days old cultures of both the microorganisms on nutrient broth diluted in 10 L water was added to the soil of one microplot. For seed treatment the broth (5ml/kg seeds) was applied on seed along with the rhizobium. Biopesticide based on *B. subtilis* was prepared and its dose was 40 g/microplot and 5 g/kg seeds.

Fungicide

Carbendazim was applied in pots @ 0.25 g a.i./pot whereas in microplots, the dose was @ 1.25 kg a.i./h which was applied in broadcast manner a week after the seed sowing, and for seed treatment the fungicide was applied @ 2g/kg seed.

Nematicide

In pots, 1.0 g a. i. fenamiphos was mixed in the soil. Soil application with fenamiphos in microplots was done @ 6 kg a.i./h and for seed treatment the dose was 2 g/kg seed.

Biopesticides

A novel process for commercial production of biopesticides has been invented. The process involved two steps. In the first step, mass culture or stock culture of biocontrol fungi and bacteria was prepared on sawdust, soil and molasses mixture. The second step involved immobilization of the biocontrol agents on a fly ash based carrier. Using the process three biopesticides based on *Trichoderma harzianum*, *Pochonia chlamydosporia* and *Bacillus subtilis* were developed. This process has not been reported earlier and the following three biopesticides have not been produced this way previously. The three biopesticides with their names and the diseases they control are as follows.

1. **Biofungicide: Biowilt-X** based on *Trichoderma harzianum* to control fusarial wilt caused by *Fusarium oxysporum* f. sp. *ciceri*.
2. **Bionematicide: Bionem-X** based on *Pochonia chlamydosporia* to control root-knot caused by *Meloidogyne incognita*.
3. **Biofunginemacide: Biocure-X** based on *Bacillus subtilis* to control fungus-nematode wilt disease complex caused by (*Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *ciceri*).

Step I: Production of mass/stock culture of bioagents

Various agricultural and waste materials viz., seed husk-soil-molasses, sawdust-soil-molasses, bagasse-soil-molasses, leaflitter-molasses, sorghum meal-molasses and sorghum seeds were tested for mass production of biocontrol fungi and bacteria. Based on relative performance of the materials tested, a mixture of sawdust-soil-5% molasses (15:5:1) was selected to develop mass (stock) culture of *T. harzianum*,

P. chlamydosporia and *B. subtilis*. One kg of the mixture was filled in heat resistant polybags, sealed and steam sterilized at 15 kg/cm^2 pressure at 121°C for 15 minutes. For *T. harzianum* and *P. chlamydosporia,* chloramphenicol 10 mg/kg material was added to the 1 kg material. Thereafter the bags containing 1 kg autoclaved sawdust-soil-molasses mixture were inoculated with homogenized pure culture of the biocontrol agents (5 ml/bag) by sterilized needle and syringe. The puncture made in the polybag to insert the needle was resealed with cello tape. Pure cultures of *T. harzianum* and *P. chlamydosporia* were prepared in potato dextrose broth supplemented with 10 mg chloramphenicol/litre, and *B. subtilis* in nutrient broth. The bags were resealed and incubated at room temperature (25-30°C) or at 25±2°C in an incubator for 10-15 days (fungi) and 35±2°C for 5 days (bacteria) in an incubator. During incubation the bags were shaken daily for a few minutes to achieve uniform colonization by the biocontrol agents on the material. Luxuriant and uniform colonization by the microorganisms occurred within the incubation duration of 5-15 days.

Step II: Immobilization of bioagents

After preliminary screening of molasses-lignite-stillage granules, alginate-bran-fermenter biomass pellets, alginate-clay pellets, diatomaceous molasses-soil pellets, sawdust-soil-molasses fermenter biomass, seed husk-sand-molasses fermenter biomass, charcoal powder/pyrex (talc) fermenter biomass powder, fly ash fermenter biomass powder, sodium alginate pellets of liquid fermenter biomass etc., to support survival and multiplication of biocontrol fungi and bacteria four carriers viz., talc, charcoal, fine clay and fly ash were selected for further study. The stock culture of bicontrol fungi viz., *T. harzianum* and *P. chlamydosporia* was mixed in the above mentioned four carriers and 5% molasses in the ratio of 1:0:1, 1:5:1, 1:10:1 and 1:20:1 (stock culture:carrier:molasses) and supplemented with 10 mg chloramphenicol/kg formulation. Thereafter, the mixture was incubated at 25±2°C in an incubator for 10 days. After incubation CFU load/g formulation was determined using the dilution plate method.

Final composition of the biopesticides

A mixture of fly ash, soil (loam) and 5% molasses in the ratio of 15:3:1 plus chloramphenicol 10 mg/kg formulation for biocontrol fungi was used as a carrier to immobilize *T. harzianum, P. chlamydosporia* and *B. subtilis* (Fig. 4).

Figure 4. Ingredients of the biopesticides developed

The fly ash was collected from a coal fired thermal power station, Kasimpur, Aligarh; where bituminous coal is burnt. Some of the important physico-chemical characteristics of the ash were: pH 8.9, conductivity 7.6 m mhos/cm, cation exchange capacity 9.3 m mhos/cm, sulphate 9.72%, carbonate 1.07%, bicarbonate 2.6%, chloride 1.85%, nitrogen 0.0%, phosphorus 0.093%, potassium 0.82%, calcium 1.06%, magnesium 0.90%, manganese 64.5 mg/g, copper 117.8 mg/g, zinc 85.1mg/g and boron 198.5 mg/g. The ash-soil mixture was solarized under thin and transparent polythene sheet for 3-4 weeks (+38°C ambient temperature) or filled in heat resistant polybags and autoclaved at 15 kg/cm^2 pressure at 121°C for 15 minutes. Thereafter,

Figure 5. Biopesticides in 200, 500 and 1000 g packets

stock culture, carrier and 5% molasses were mixed in the ratio of 1:5:1, 1:10:1, 1:15:1 and 1:20:1 and filled in polybags. The bags were sealed and incubated for 10-15 days at room temperature (25-35°C) or inside an incubator at 25±2°C for *T. harzianum* and *P. chlamydosporia*, and 35±2°C for *B. subtilis*. After incubation number of colony forming units (CFUs)/g formulation was determined using dilution plate method. The formulations were packed in airtight polypacks of 200, 500 and 1000 g (Fig. 5).

Shelf life

Shelf life of the three biopesticides was tested at five temperature regimes i.e., 5°C, 10°C, 15°C, 25°C and ambient (February to May) for 32 weeks. The viability and CFU load was determined weekly.

Crop culture (Chickpea)

Certified seeds of chickpea, *Cicer arietinum* (L) cv. BG-256 were procured from an authorized dealer. The mycoflora examination of seeds (external and internal) through blotter paper test (Tempe, 1970) revealed absence of *F. oxysporum* f. sp. *ciceri* or other potential pathogenic fungi on the seeds. The seeds were sown in pots (5 seeds/pot) and in rows in microplots (57 seeds/row, 4 rows/microplot) where antagonists had already been applied. The BG-256 matures in 120 days. One week after sowing irrigation was done. Hence, the plants were allowed to grow for four months. During this period they were regularly observed for any symptom.

Soil population

Fungi and bacteria

Soil population of the wilt fungus (*F. oxysporum* f. sp. *ciceri*) and biocontrol agents (*T. harzianum*, *T. virens*, *P. chlamydosporia*, *B. subtilis and P. fluorescens*) was estimated monthly using dilution plate method (Waksman, 1927). Soil was collected from the rhizosphere of the plants from pots and microplots and was mixed together to make a composite sample. One gram of the soil was taken in a conical flask to which 9 ml sterile water was added. The soil-water mixture was stirred over a magnetic stirrer for 5 minutes. One ml of this suspension was transferred to 9 ml sterile water in a test tube. One ml sample was then transferred to another tube containing 9 ml sterile water. The process was repeated till the desired dilution of

1: 100000 (for fungi) and 1: 10000000 (for bacteria) was achieved. Each suspension was shaken over magnetic stirrer for few seconds and was in motion while being drawn into the micropipette. From the final dilution 0.1 ml suspension was aseptically spread under Laminar flow over solidified *Fusarium* specific medium (Appendix 23), *Trichoderma* specific medium, *P. chlamydosporia* semi-selective medium (Appendix 2, 3), Maintainance Medium and King's B with various supplements (Appendix 5) in Petri plates and three plates were maintained for each dilution. The agar plates were prepared four days earlier to ensure that the medium in the plate was free from contamination. After inoculation the plates were then incubated at 27±2°C for 5-10 days for fungi and 35±2°C for 2 days for bacteria to get the colonies. After incubation, the plates were examined under a colony counter to determine soil population of the microorganism on the basis of morphological and PCR-RAPD tests.

RAPD-PCR study on genomic DNA of biocontrol agents

To match the identity of the populations of *Trichoderma harzianum* , *Pochonia chlamydosporia* and *Bacillus subtilis* recovered from the soil with those applied in the soil and to distinguish them from the indigenous (local) populations of the microorganisms, a preliminary molecular study (RAPD-PCR) was done. In this study ten primers, 5 of Operon series A (OPA-6, OPA-7, OPA-8, OPA-9, OPA-10) and 5 synthetic primers were used to amply the template DNA. This assay was done with the help of kind infrastructural support and guidance at RRL, Jammu, India.

Isolation of genomic DNA from *Trichoderma harzianum* and *Pochonia chlamydosporia*

From the plates of serial dilution method used to count soil population of *T. harzianum* and *P. chlamydosporia*, morphologically similar 5 colonies were randomly picked to isolate genomic DNA. The vacuum dried fungus mycelium (3 g fresh or frozen) was crushed in 15 ml of Grinding Buffer (10ml 3M sodium acetate; 37.5 ml 4M NaCl; 30 ml 0.5 M EDTA, pH 8.0; 15 ml 1 M Tris Cl; 6 g PVP; 4.2 g SDS and double distilled water to make volume 300 ml) using mortar and pestle. The resultant paste was transferred to sterile centrifuge tubes using sterile spatula. The tube was incubated in water bath at 60-65°C for one hour with gentle inversion

at about every 15 min. Thereafter 3 ml of 10 M ammonium acetate was added in to the tube and kept for another 30 min at 65°C followed by centrifugation at 10,000 rpm for 10 min. The supernatant was transferred to an other tube to which an equal volume of chilled isopropanol was added and kept at -20°C for 60 min or 0°C over night. The DNA was pelleted out by centrifugation (6,000 rpm for 25 min or 10,000 rpm for 15 min at 4°C), washed twice with 70% ethanol and dissolved in $T_{10}E_1$ buffer (1ml 1 M Tris; 200 µl 0.5 M EDTA, pH 8.0; double distilled water to make volume 100 ml). Dissolved DNA solution was extracted with chloroform: iso-amyl alcohol (24:1) and RNA was removed by RNAse (enzyme) treatment (@ 4 µl/ml of supernatant from stock of 10 mg/ml of RNAse) at 37°C for one hour. RNAse treated DNA was further extracted twice with chloroform: iso-amyl alcohol (24:1) for further purification. DNA was re-precipitated in chilled ethanol (100%) and dissolved in $T_{10}E_1$ buffer. Purified DNA was checked for its quality and quantity by 0.8% agarose gel electrophoresis using uncut lambda-DNA as standard (300 ηg/µl). Dilution of the DNA solution was done using $T_{10}E_1$ buffer to a concentration of approximately 25 ηg/2µl for use in PCR analysis.

Isolation of genomic DNA from *Bacillus subtilis*

Five milliliters of brain heart infusion (BHI) broth was inoculated with each of the 5 morphologically similar colony of *Bacillus subtilis*, and the cultures were incubated overnight with shaking at 30°C. Bacterial cells were harvested by centrifugation at 1,000 x g for 5 min, and bacterial pellets were rinsed with sterile water. DNA was isolated by using the Wizard genomic DNA purification kit (Promega, Madison, USA) according to the protocol provided by the manufacturer. DNA quantity and quality were determined by electrophoresis with amount of lambda DNA, marker VI (Roche S.p.a., Milan, Italy) as a standard.

Amplification of DNA of *Trichoderma harzianum* and *Pochonia chlamydosporia* by polymerase chain reaction

PCR amplification was carried out in 0.2 ml thin-wall PCR tubes using a PCR machine thermal cycler (model T1 Thermocycler, Biocycler, Elosen, Riga Latvia). A total of 10 RAPD primers were screened in the study. Five primers were Operon series A (OPA-6 to OPA-10, Operon Technologies, Almeda, CA, USA) and rest five primers were fungus specific (Primer 1- GTCACCCGGA; Primer 2- GCGACGCCTA; Primer

3- GCGGCATTGT; Primer 4- AGTGGTCGCG; Primer 5- CCAGACAAGC) custom synthesized from Genetix Biotech Asia Pvt. Ltd., India. An unambiguous DNA profile was generated by 5 primers (OPA9- CGGTAACGCC and Primer 1, Primer 3, Primer 4, Primer 5) and 6 primers (OPA9 and Primer 1, Primer 2, Primer 3, Primer 4, Primer 5) in case of *Trichoderma harzianum* and *Pochonia chlamydosporia,* respectively. Polymerase chain reaction (PCR) mixture of 25 µl contained 25 ηg of genomic DNA templates, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 µM of decamer primer, 2.5 µl of 10x PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$) and 0.25 µl of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, USA). PCR cycle conditions were as follows: initial denaturating step at 94°C for 3 min followed by 44 cycles of 92°C for 1 min, 37°C for 1 min and 72°C for 2 min. In the last cycle, primer extension at 72°C for 7 min was provided.

Amplification of *Bacillus subtilis* DNA by polymerase chain reaction

PCR amplification was carried using a PCR machine thermal cycler (model T1 Thermocycler, Biocycler, Elosen, Riga Latvia). A total of ten RAPD primers were tried, five from Operon series OPA-6 to OPA-10 (Operon Technologies, Almeda, CA, USA) and five synthetic primers. An unambiguous DNA profile was generated by 4 primers ((Primer 6- GGAGCGGTCG; Primer 7- CTATCAGCAC; Primer 8- CGTGCCGATT; Primer 9- CGCTATCAAT). Polymerase chain reaction (PCR) mixture of 25 µl contained 25 ηg of genomic DNA templates, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 µM of decamer primer, 2.5 µl of 10X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$) and 0.25 µl of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, USA). PCR cycle conditions were followed as described by Matarante *et al.* (2004). Amplification consisted of an initial denaturation at 94°C for 1 min, followed by 20 cycles of 94°C for 30 s. 29°C for 1 min, 72°C for 1 min and 40 additional cycles of 94°C for 30 s, 55°C for 40 s, and 72°C for 30 s; the annealing and extension of the last 40 cycles were elongated for 1 and 2 s per cycle, respectively. A final extension at 72°C for 5 min was added.

Documentation of gel and data analysis

PCR products were electrophoretically separated on a 1.5% agarose gel containing ethidium bromide [@ 1 µl (10 mg/ml) per 250 ml agarose solution] using 1x TBE

buffer (pH 8.0) and 60 V current was run for three hours. The amplified product were visualized and photographed under UV light source. O' Gene Ruller™ 100 bp DNA Ladder Plus (Fermentas Life Sciences, USA) was used as molecular weight marker.

DNA bands were scored '1' for its presence and '0' for its absence for each primer-genotype combination. These binary data matrix was then utilized to generate genetic similarity data among genotypes. Only unambiguous bands were scored for the estimation of genetic similarity between the isolates using Jaccard's similarity coefficient. Based on these data dendrograms were generated by using the SAHN clustering programme selecting the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Nei and Li, 1979) in NTSYS-pc (Rohlf, 1992).

Root-knot nematode

Population of *Meloidogyne incognita* was determined by Cobb's decanting and sieving method (modified) followed by the Baermann funnel technique (Southey, 1986). A composite soil sample was made by collecting the soil from three randomly selected pots and rhizosphere of five plants in each microplot in case of field trials. The soil was sifted through a coarse sieve. The soil sample (1 kg) was mixed in 5 litres of water in a plastic bucket. The soil-water mixture was stirred and then allowed to stand for 1-2 minutes. The suspension was decanted over a combination of 3 sieves (60, 200 and 500 mesh), the catch from the final sieve was carefully washed and transferred to a beaker.

A small coarse sieve with two layers of wet paper towels was kept in a Baermann funnel filled with water. The nematode suspension from the beaker was gently poured onto the sieve and allowed to stand overnight. The nematode juveniles because of the random and continuous movement migrate through the paper pores into the water and gradually settled down in the bottom of rubber tubing of the funnel. The nematode suspension recovered from the Baermann funnel was taken into a beaker and counted in a counting dish under a stereomicroscope.

Root-nodulation

Two month old plants were randomly selected from three pots (out of six) and were uprooted carefully to observe nodulation. From each microplot 5 plants (2 months old) were randomly uprooted to count nodules on the root. Pink and healthy nodules

were recognized as functional nodules, where as dark brown and degenerated ones as non-functional nodules.

Wilt incidence and severity

Two months old plants of chickpea were visually observed to determine wilt severity 0-5 scale and incidence (%) according to the following formulae:

$$\text{Wilt incidence (\%)} = \frac{\text{Number of wilted plants in a microplot}}{\text{Total number of plants in a microplot}} \times 100$$

$$\text{Wilt severity} = \frac{\text{Number of branches / twigs showing wilt symptom}}{\text{Total number of branches / twigs of a plant}} \times 100$$

The severity was converted into wilt index on 0-5 scale. 0= no wilting, 1= 1-19%, 2= 20-39%, 3= 40-59%, 4= 60-79%, 5= 80-100%.

Harvesting

Four months after sowing, chickpea plants were harvested from pots (3 plants/ treatment) whereas in case of field trials, 10 plants from each microplot were randomly uprooted and the following parameters in addition to nodulation and wilting were determined.

1. Root-knot severity
2. Nematode reproduction
3. Dry weight of plants
4. Weight of seeds/plant
5. Soil population of pathogens and biocontrol agents

Root-knot

Slow stream of water was used to wash the root system of the matured harvested plants. The roots were stained with phloxine B solution (0.159 g/l) in order to count egg masses and galls.

Statistical analysis

Three replicates were maintained for each treatment and the observations taken from ten plants from a microplot were averaged and considered as one replicate. For pot experiments, 6 replicates were maintained for each treatment with additional 9 replicates for nematode treatments which were used to estimate nematode population in pot soil. The data on dry matter production and yield was subjected to a two factor analysis of variance. Pathogens were considered as one factor, whereas control treatments as second factor. The data on wilt incidence, root knot, soil population etc. were analyzed for single factor ANOVA. Wilt incidence was angularly transformed before the analysis. Least significance difference (L.S.D.) was calculated at $P \leq 0.05$ for all variables to compare individual treatments. The data has been presented in tabulated and graphical forms.

RESULTS

Experiment - I

In-vitro experiment

Characteristics of wilt fungus, *Fusarium oxysporum* f. sp. *ciceri*

The wilt causing fungus was identified on the basis of cultural and morphological characteristics. The fungus grew upto 70 mm in 5 days on potato dextrose agar (PDA) and produced hyaline cottony mycelium, later it became pale yellow (Fig. 6). The mycelium was septate and produced three types of spores. Microconidia were small, elliptical or with 1-2 septa, whereas the macroconidia were long or curved (fusaroid) (Fig. 6). Chlamydospores were oval or spherical and formed in older cultures from any cell of the hyphae.

To fulfill Koch's postulates, the inoculum of *F. oxysporum* f. sp. *ciceri* was prepared on sorghum grains and inoculated in pots containing autoclaved soil. Healthy and surface sterilized seeds of chickpea cv. BG-256 were sown and the symptoms of disease were observed 30 days after sowing. The wilt symptoms were identical to those recorded in naturally infected chickpea plants. Blackening was sometimes visible through the bark as streaks or bands (Fig. 6).

Characteristics of biocontrol agents

***Trichoderma harzianum* (Th 00)**

T. harzianum is a fast growing fungus and it covered the entire 90 mm dia. PDA petri plate in 4 days. It produced septate, hyaline and branched mycelium. The fungus produced spores within 2 days of incubation in alternate concentric rings. Spores were formed on divergent whorls of 2-6 phialides borne on conidiophores. The phialospores were green, sub globose to short ellipsoid 1.5-2.5 mm in size. Chlamydospores were also formed present intercalary in the mycelium. The fully grown PDA plate was light green with fluffy mycelial growth (Fig. 7 & 8).

***Trichoderma virens* (Tv 00)**

The fungus also grew actively and attained a growth of 70-80 mm dia in 4 days. Aerial mycelium was floccose initially white and later turned deep green due to sporulation. It produces conidiophores terminated with flask shaped convergent

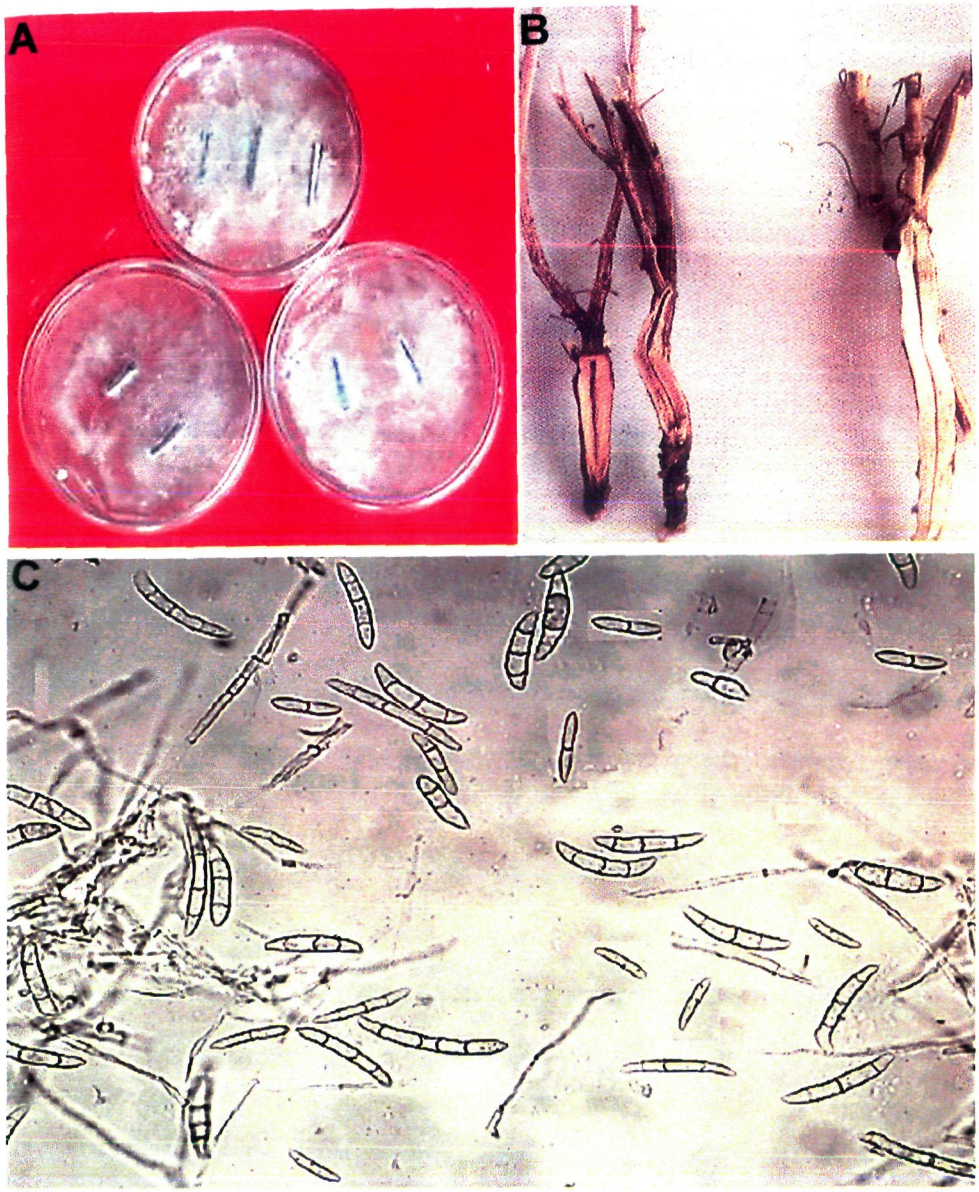


Figure 6. The pathogen *Fusarium oxysporum* f. sp. *ciceri*. Colonies from infected tissue (A); vascular browning (B); micro and macroconidia (C).

phialides. Conidia from adjacent phialides often coalesce into large gloeoid masses (spore ball) (Fig. 7 & 8).

Pochonia chlamydosporia

P. chlamydosporia is a slow growing fungus and covered 30 mm dia growth in 10 days on PDA. Colonies appeared granular due to the abundant production of dictyochlamydospores which were 15-20 mm in size. The mycelium was white in colour (Fig. 7 & 8).

Bacillus subtilis

The bacteria produced milky white colonies with rough or sereated margins. The colonies smell earthern. Cells were rod shaped or straight (Fig. 7 & 8). It stained violet on performing Gram staining test. The cells in four days old culture possessed endospores. The biochemical and physiological characterization of the strain and soil isolates of *B. subtilis* have been presented in Table (16) .

Pseudomonas fluorescens

The bacteria produces slimy colonies on the King's B Agar medium with smooth margins (Fig. 7 & 8). It produced fluorescein pigments into the medium. Cells were straight rods and the bacteria stained pink on Gram staining showing its gram -ve nature. The biochemical and physiological characterizations of the strain and soil isolates of *P. fluorescens* have been presented in Table (16) .

Comparison of growth rates (monoculture)

Monoculture of fungal biocontrol agents revealed that *T. harzianum* isolate/strain showed the faster radial growth and covered the entire PDA plate (90 mm dia.) within 48 hrs of inoculation and incubation followed by *T. virens* (81 mm). Other soil isolates of *Trichoderma* spp. also grew luxuriantly but were less efficient than standard strains (Fig. 9).

Dual culture test

Dual culture tests revealed that *T. harzianum* and *T. virens* overgrew on *F. oxysporum* f. sp. *ciceri* mycelium and caused 100% inhibition after 120 hrs. (Fig.10& 11). The dual culture tests with *P. fluorescens* and *B. subtilis* conducted

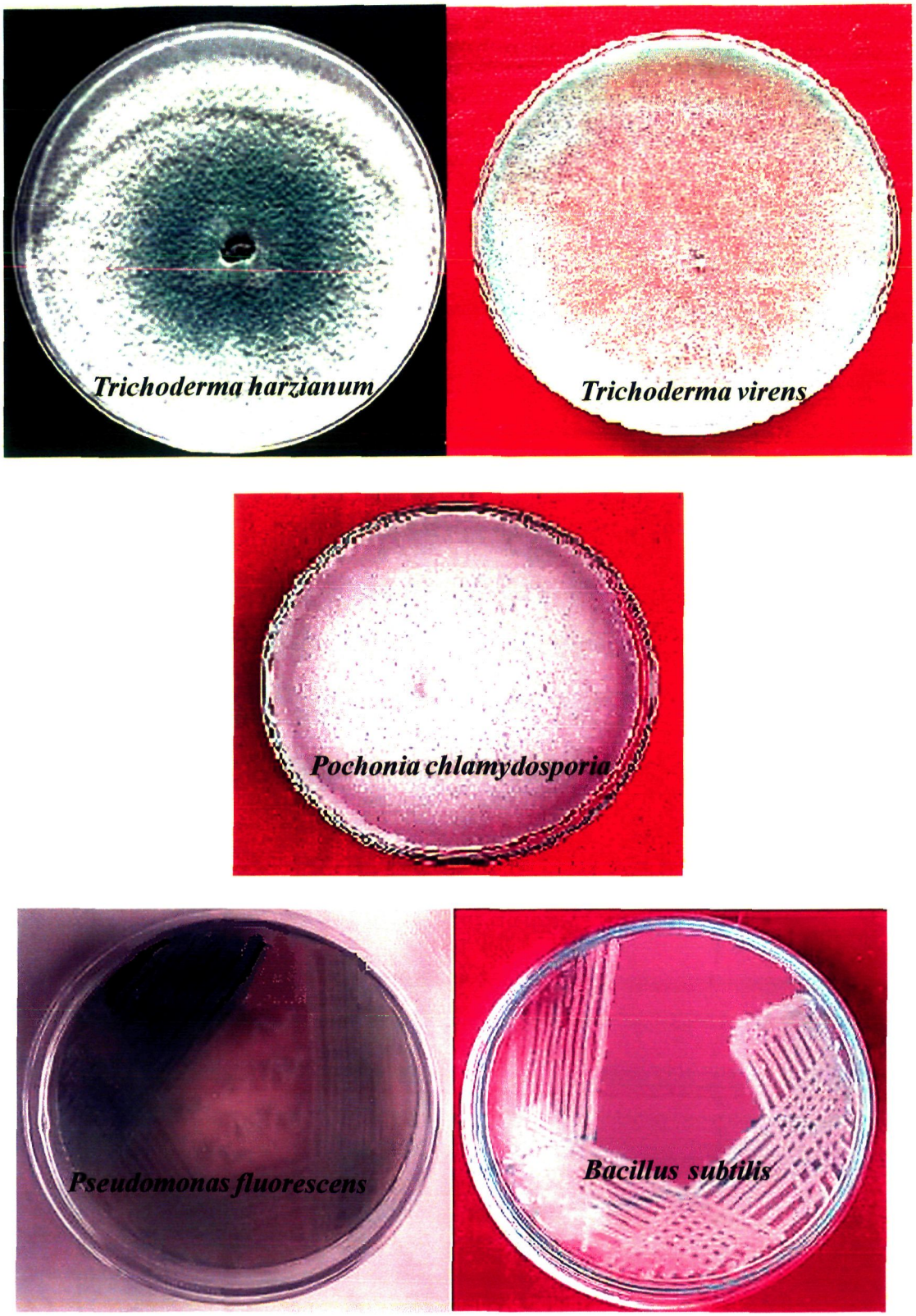


Figure 7. Colony characters of biocontrol fungi and bacteria

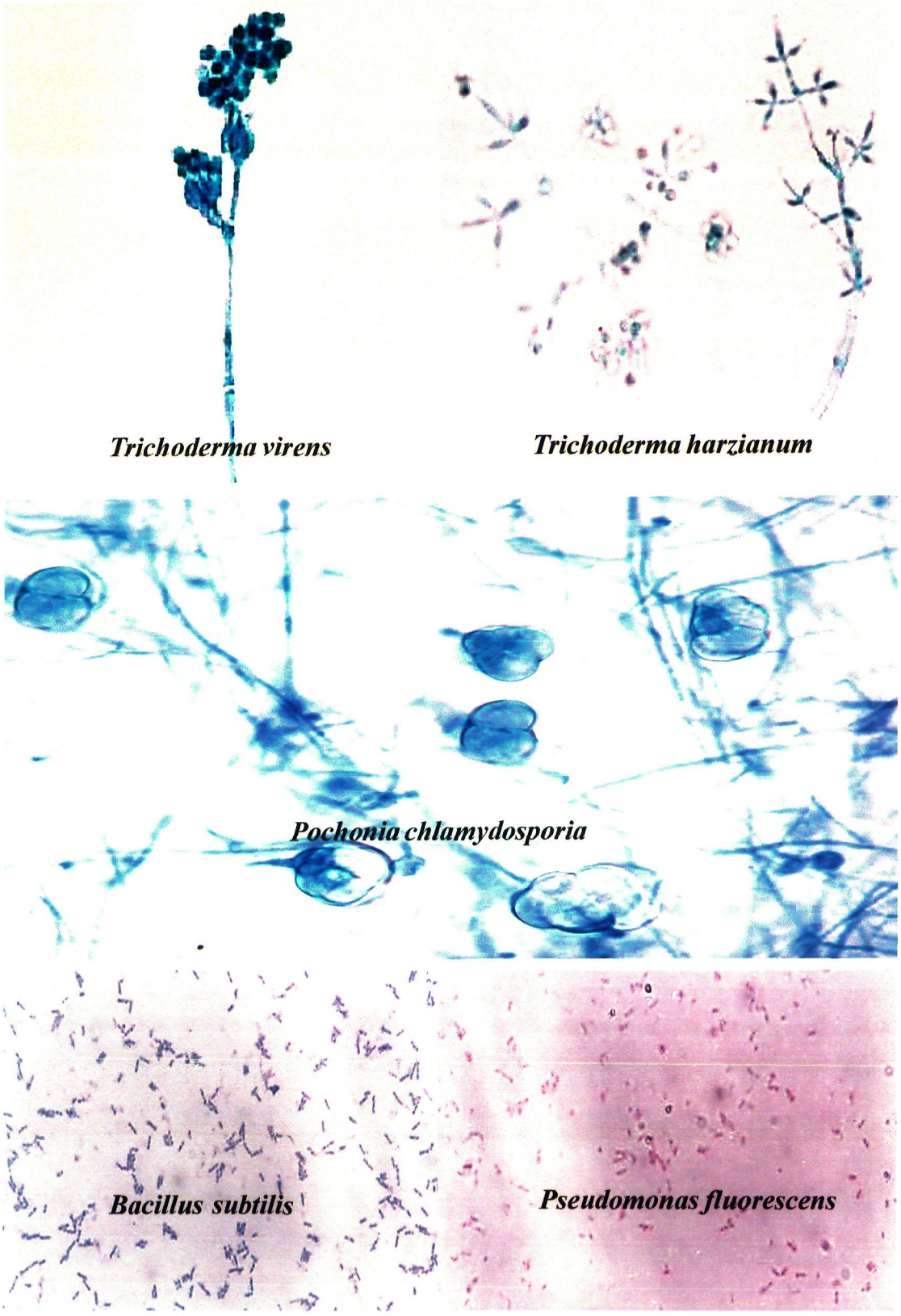


Figure 8. Morphological characters of biocontrol fungi and bacteria

Table 16. Biochemical and physiological characterization of some strains/isolates of *Bacillus subtilis* (Bs) and *Pseudomonas fluorescens* (Pf).

Test	Bs00*	Bs01	Bs02	Bs03	Bs04	Bs05	Pf00*	Pf01	Pf02	Pf03	Pf04	Pf05
Fermentation,												
glucose	+	+	+	+	+	+	+	+	+	+	+	+
fructose	+	+	+	+	+	+	+	+	-	-	+	+
maltose	+	-	-	-	-	-	-	-	-	-	-	-
sucrose	+	+	+	-	-	+	+	-	-	-	-	-
Ammonification	-	-	-	-	-	-	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	-	-	+	-
Indole	+	-	-	-	-	-	-	-	-	-	-	-
Levan formation	.....NA.....						+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-	+	+	+	+	+	+
Starch hydrolysis	+	+	-	-	-	-	+	-	-	+	+	-
Tripple sugar iron agar test	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer test	+	-	-	-	-	+	-	-	-	-	-	-
HCN production	-	-	-	-	-	-	+	+	+	+	+	+
Antifungal activity	+	+	+	+	+	+	+	+	+	+	+	+
Indole acetic acid production	+	+	+	+	+	+	+	+	+	+	+	+
Phosphorus solubilization	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4C	.....NA.....						+	+	+	+	+	+
Growth at 41C	.....NA.....						-	-	-	-	-	-
Fluorescent diffusable pigments	.....NA.....						+	+	+	+	+	+

* Standard strains otherwise soil isolates; + Tests showing positive response; - Tests showing negative response; NA Not applicable.

Figure 9. Radial growth of strains/isolates of biocontrol fungi in monoculture *in-vitro.* Th 00/ Tv 00 Standard isolate, Th 01-05/Tv 01-05 Soil isolates.

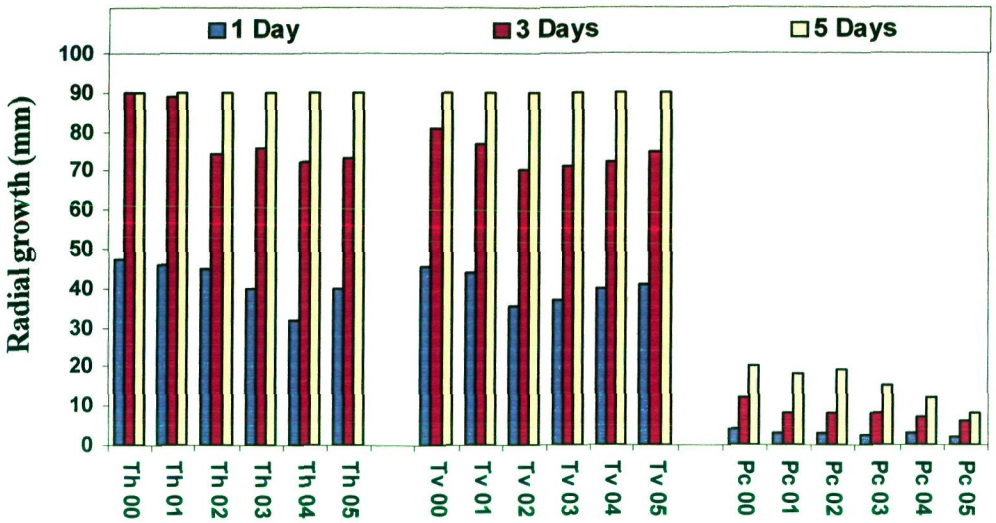
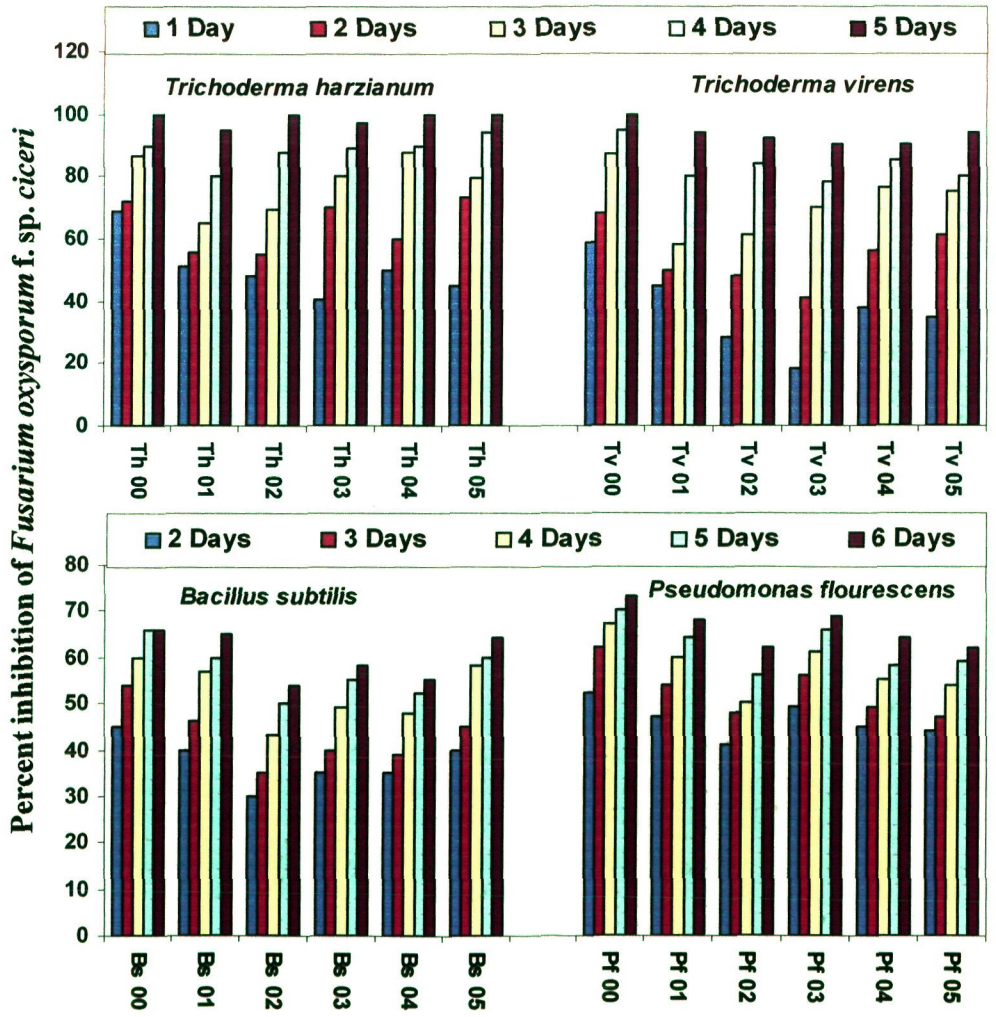


Figure 10. Inhibition of *Fusarium oxysporum* f. sp. *ciceri* in dual culture test with strains/ isolates of biocontrol agents *in vitro*. Th 00/Tv 00/Bs 00/Pf 00 Standard isolate, others soil isolates.



in the present study have shown 52-73% and 45-66% inhibition in the growth of *F. oxysporum* f. sp. *ciceri* during 48-144 hrs (2-6 days), respectively (Fig. 10 & 11). Other soil isolates also inhibited the growth of wilt fungus but were inferior than the standard strains (Fig. 10 & 11).

Mycoparasitism

Trichoderma spp. interacted readily with the mycelium of *F. oxysporum* f. sp. *ciceri* as was evident by the overgrowth and lysis of mycelium of the pathogenic fungus. Microscopic examination of mycelium from the zone of interaction revealed parallel running and coiling of *F. oxysporum* f. sp. *ciceri* mycelium by the mycelium of *Trichoderma* spp. Standard strains of *T. harzianum* and *T. virens* were more efficient than other soil isolates as they completely utilize the *Fusarium* mycelium in 5 days on PDA plates (Fig. 10 & 11).

Effect of volatile compounds

Effects of 2, 4, 8 and 10 days old cultures of *Trichoderma* spp. were tested against *F. oxysporum* f. sp. *ciceri* and an inverse relationship between the age of *Trichoderma* culture and amount of volatile compound production was recorded. Consequently more inhibition in the radial growth of *F. oxysporum* f. sp. *ciceri* was observed with fresh cultures. Volatile compounds produced by 48 hour old isolates of *Trichoderma* spp. significantly ($P \leq 0.05$) reduced radial mycelial growth of *F. oxysporum* f. sp. *ciceri*. Maximum inhibition of the mycelial growth of *F. oxysporum* f. sp. *ciceri* (75%) was recorded by *T. harzianum* followed by *T. virens* (71%). The suppressive effects on the growth of the pathogen, however, decreased ($P \leq 0.05$) with the age of cultures being lowest with 10 days old cultures (Fig. 12 & 14).

Effect of non-volatile compounds (culture filtrates)

When *F. oxysporum* f. sp. *ciceri* was grown on the PDA amended with 10, 30 and 50% concentrations of culture filtrates of biocontrol agents, radial growth of the pathogen was inhibited compared to PDA without culture filtrate (Fig. 13 & 14). Maximum decrease (75%) in the radial growth of *F. oxysporum* f. sp. *ciceri* was recorded with *T. harzianum* culture filtrate with the concentration of 50% followed by the same concentration of *T. virens* (65%) (Fig. 13).

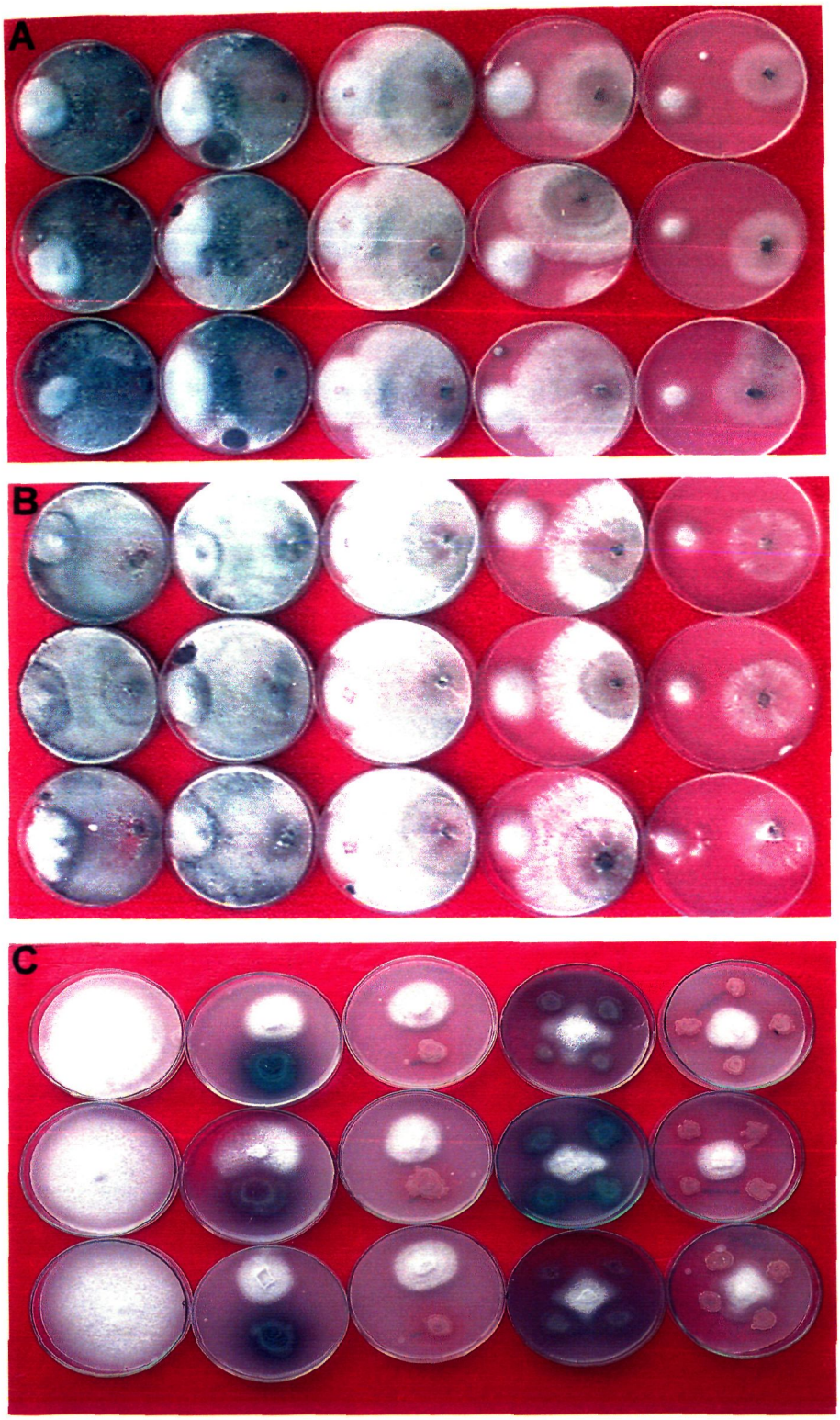


Figure 11. Antifungal activity of *Trichoderma harzianum* (A), *Trichoderma virens* (B), and *Bacillus subtilis* and *Pseudomonas fluorescens* (C) against *Fusarium oxysporum* f. sp. *ciceri* in dual culture test.

Figure 12. Inhibition in the colonization by *Fusarium oxysporum* f. sp. *ciceri* due to volatile compounds produced by *Trichoderma* strains/isolates *in-vitro*. Th 00/Tv 00; Standard isolate, Th 01-05/Tv 01-05; Soil isolate.

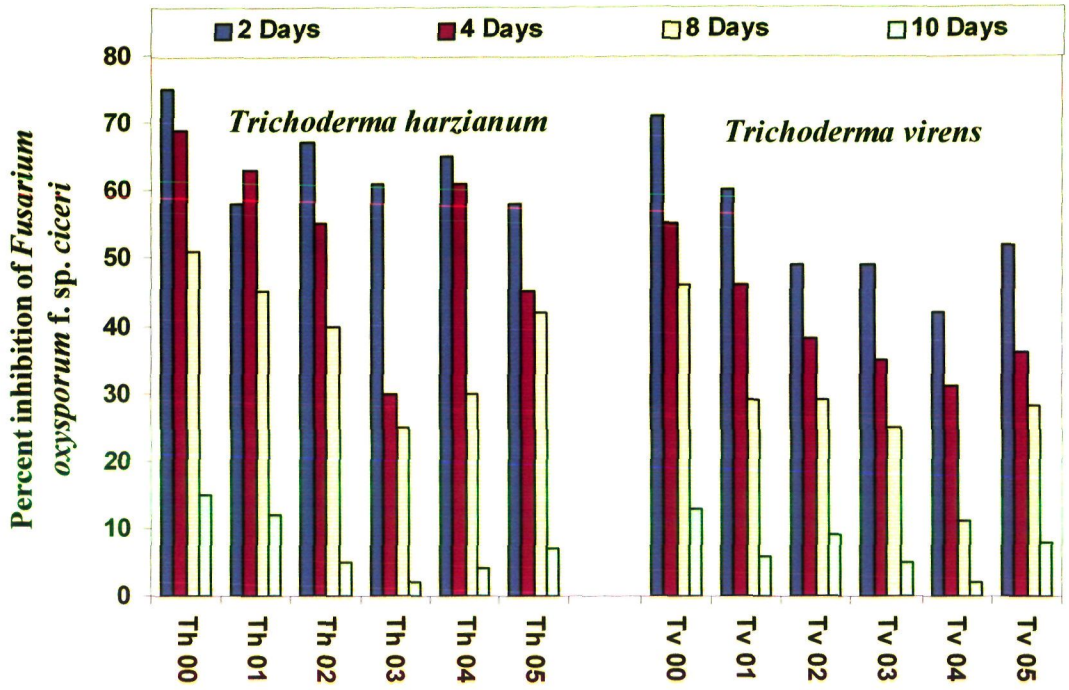
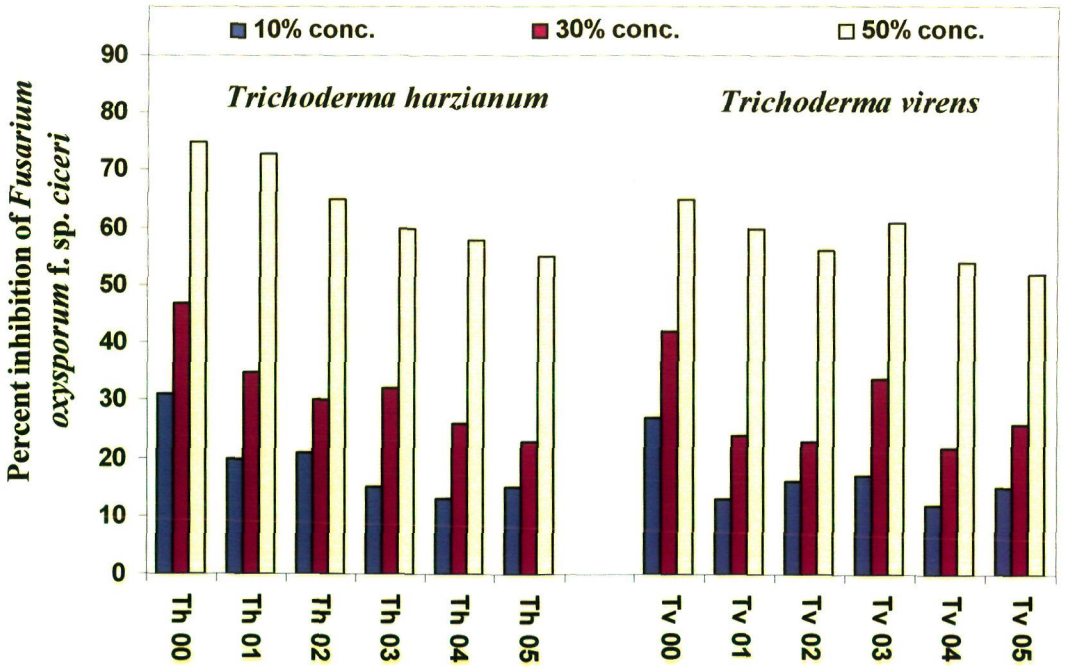


Figure 13. Inhibition in the colonization by *Fusarium oxysporum* f. sp. *ciceri* due to non-volatile compounds produced by *Trichoderma* strains/isolates *in-vitro*. Th 00/Tv 00 Standard isolate, Th 01-05/Tv 01-05 Soil isolate.



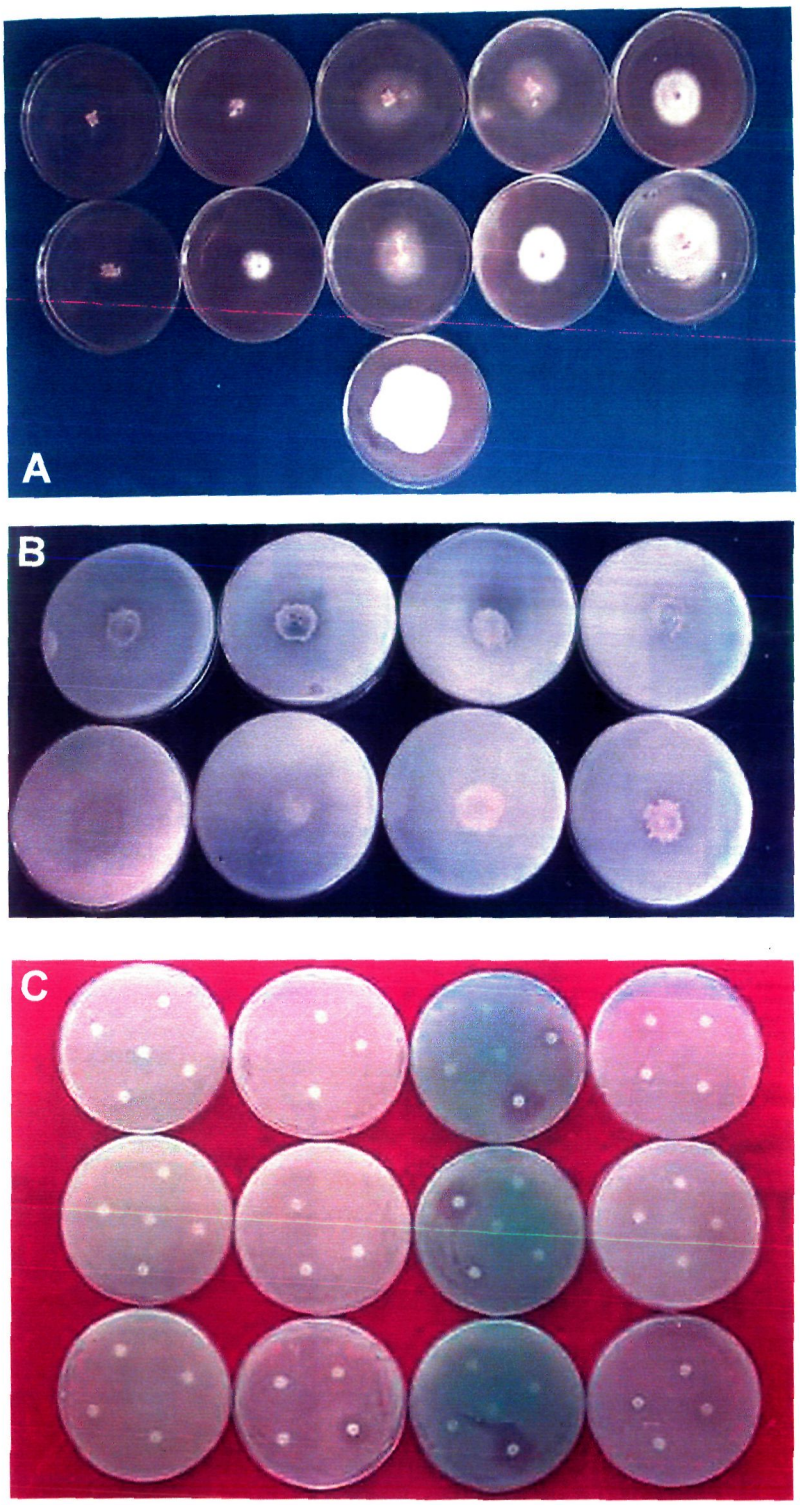


Figure 14. Effect of volatile compounds of *Trichoderma* spp. on the growth of *Fusarium oxysporum* f. sp. *ciceri* (A); phosphate solubilization (B) and antibiotic profiling (C) of biocontrol bacteria.

Nematicidal effects of biocontrol agents *in-vitro*

Egg hatching and larval mortality of *Meloidogyne incognita*

The hatching of juveniles from egg masses incubated with uninoculated broth (control) was almost identical to distilled water (Table 17). The hatching was, however, suppressed in the culture filtrates. The hatching decreased by 100% in the culture filtrate of *Pochonia chlamydosporia* compared with the control after 5 days of incubation. Treatments with the standard strains of *B. subtilis* (72-97%), *P. fluorescens* (50 - 84%), *T. harzianum* (48 - 80%) and *T. virens* (40 - 71%) inhibited the hatching. The culture filtrates also induced mortality to the juveniles of *M. incognita* (Table 18). Mortality in the juveniles was 65 and 100% with the culture filtrate of *P. chlamydosporia* within 12 and 24 hrs of incubation. Percent mortality due to *B. subtilis* filtrate was 55, 76 and 90% within 24, 48 and 96 hrs. of incubation, respectively (Table 18).

Antibiotic profiling of biocontrol bacteria

Antibiotic profiling was done for the used strains of soil bacteria against 12 antibiotics to develop antibiotic resistant marker strain of *P. fluorescens* and *B. subtilis* (Table 19; Fig. 15). The profiling revealed 25 mg/l medium minimum inhibitory concentration (MIC) of tetracycline hydrochloride (Hi-Media, India) for *P. fluorescens*; from this MIC, minimum tolerance concentration (MTC) of tetracycline i.e., 20 mg/l was determined (Table 20). Resistant marker strain of *P. fluorescens* was developed by subjecting the culture successively from low to high concentrations of tetracycline. Tetracycline resistant (20 mg/l) strain of *P. fluorescens* thus obtained was used throughout the study. For the isolation of this bacteria strain from soil, 20 mg tetracycline/l medium was added to Kings B medium to make the medium specific for the tetracycline resistant marker strain of *P. fluorescens*. The MIC could not be determined for *Bacillus subtilis* as the strain was found sensitive to all antibiotics tested.

Compatibility of pesticides with biocontrol agents

The compatibility test of the *Trichoderma* spp. revealed that the maximum growth of the fungi was inhibited (ED_{90}) at concentrations 405 and 500 µg carbendazim/ml, 2200 and 2400 µg metalaxyl/ml, 875 and 1000 µg captan/ml, 625 and 755 µg mancozeb/ml, 95 and 150 µg thiram/ml and 1000 and 1150 µg nemacur/ml

Table 17. *In vitro* effects of culture filtrate of biocontrol agents on hatching of egg masses of *Meloidogyne incognita* incubated for 5 days.

Treatments	Distilled water	Broth alone	Culture filtrate		
			25%	50%	100%
Trichoderma harzianum	210	198	102	97	40
T. virens	210	198	119	105	58
Pochonia chlamydosporia	210	198	02	00	00
Bacillus subtilis	210	200	55	11	05
Pseudomonas fluorescens	210	200	100	83	32

Values are number of juveniles hatched per egg mass; Each value is mean of three replicates.

Table 18. *In vitro* effects of culture filtrates of biocontrol agents on mortality of *Meloidogyne incognita* juveniles.

Treatments	Incubation hours	Percent Mortality		
		Distilled water	Broth alone	Culture filtrate
Trichoderma harzianum	12	-	-	-
	24	-	-	-
	48	-	04	30
	96	-	04	45
T. virens	12	-	-	-
	24	-	-	-
	48	-	04	15
	96	-	10	20
Pochonia chlamydosporia	12	-	-	65
	24	-	-	100
	48	-	04	100
	96	-	10	100
Bacillus subtilis	12	-	-	-
	24	-	-	55
	48	-	-	76
	96	-	05	90
Pseudomonas fluorescens	12	-	-	-
	24	-	-	42
	48	-	-	53
	96	-	05	65

Each value is mean of three replicates.

Table 19. Sensitivity of *Pseudomonas fluorescens* and *Bacillus subtilis* strains/isolates to some common antibiotic drugs.

Antibiotics and potency (μg)	*Pseudomonas fluorescens*						*Bacillus subtilis*					
	Pf00	Pf01	Pf02	Pf03	Pf04	Pf05	Bs00	Bs01	Bs02	Bs03	Bs04	Bs05
Nalidixic acid 30	R	R	R	R	R	R	17*	20	17	22	18	20
Tetracycline 30	R	R	R	R	R	R	21	26	25	26	23	25
Doxycycline 30	R	R	R	R	R	R	23	25	25	25	24	26
Nitrofurantoin 30	R	R	R	R	R	R	17	17	20	23	20	22
Cotrimoxazole 30	R	R	R	R	R	R	29	33	31	33	29	25
Novobiocin 30	R	R	R	R	R	R	32	33	32	33	35	34
Flucanazole 10	R	R	R	R	R	R	28	30	30	28	33	22
Amoxicillin 30	R	R	R	R	R	R	36	36	36	33	35	26
Cloxacillin 30	R	R	R	R	R	R	24	27	25	26	24	25
Penicillin 6	R	R	R	R	R	R	36	38	40	38	42	39
Methicilline 5	R	R	R	R	R	R	30	33	35	35	34	32
Chloramphenicol 30	15	19	21	22	17	20	30	35	33	35	36	32

Each value is mean of three replicates; Pf00/Bs00 Standard strains; Pf01-05/ Bs01-05 soil isolates; R Resistant; * Inhibition zone in mm.

Table 20. Minimum inhibition (MIC) and maximum tolerance (MTC) concentrations (mg/l) of some antibiotic drugs for *Pseudomonas fluorescens* strains/isolates.

Antibiotic	Minimum inhibition conc. (MIC)						Maximum tolerance conc. (MTC)					
	Pf00	Pf01	Pf02	Pf03	Pf04	Pf05	Pf00	Pf01	Pf02	Pf03	Pf04	Pf05
Amoxycillin	200*	245	220	195	200	210	195	240	215	190	150	205
Novobiocin	50	52	48	57	58	48	45	47	43	52	53	43
Penicillin	80	77	86	80	85	88	75	72	81	75	80	83
Chloramphenicol	10	15	13	17	12	10	5	10	8	12	7	5
Tetracycline	25	23	29	25	24	20	20	18	24	20	19	15
Cotrimexazole	28	25	23	21	32	31	23	20	18	16	27	26
Doxycycline	20	26	22	18	23	25	15	21	17	13	18	20
Methicilline	77	75	83	73	70	74	72	70	78	68	65	69
Flucanazole	37	33	35	42	32	35	32	28	30	37	27	30
Nitrofurantoin	23	23	28	26	29	21	18	18	23	21	24	16

Each value is mean of three replicates; Pf00: Standard strain of *Pseudomonas fluorescens*; Pf01-05 soil isolates; R Resistant; * Concentration of antibiotics in mg/litre.

(Table 21). Tolerance for metalaxyl was 5 times higher than carbendazim, 2400 µg metalaxyl/ml and 500 µg carbendazim/ml medium inhibited 90% (ED_{90}) growth of *T. harzianum.* The fungicides at concentrations of 60 µg carbendazim/ml, 160 µg captan/ml, 225 µg mancozeb/ml, 1050 µg metalaxyl/ml and 980 µg nemacur/ml seem to be safe tolerance limit (ED_{50}) for *T. harzianum.* For *T. virens,* ED_{50} was 40 µg carbendazim/ml, 125 µg captan/ml, 177 µg mancozeb/ml 1000 µg metalaxyl/ml and 700 µg nemacur/ml. The ED_{50} of thiram for growth of *T. harzianum* and *T. virens* was 150 and 95 µg/ml medium (Table 21). Therefore, 25 and 9 µg of thiram/ml concentrations seem to be safe tolerance limit (ED_{50}) for *T. harzianum* and *T. virens,* respectively. *Pochonia chlamydosporia* showed less tolerance to the five fungicides tested. The fungus was inhibited (ED_{90}) by the concentrations of 250 µg carbendazim/ml, 500 µg each of captan and metalaxyl/ml, 350 µg mancozeb/ml. 50 µg thiram/ml and 450 µg nemacur/ml. Whereas the safe tolerance limit (ED_{50}) values for *P. chlamydosporia* were 37.5 µg carbendazim/ml, 75 µg captan/ml, 100 µg metalaxyl/ml, 5 µg thiram/ml, 110 µg mancozeb/ml and 250 µg nemacur/ml.

Biocontrol bacteria were found more tolerant to fungicides than fungi (Table 21). The maximum tolerance concentration (MTC) for *B. subtilis* were 3200 µg captan/ml, 60 µg thiram/ml 600 µg mancozeb/ml. Whereas in case of carbendazim the bacteria showed tolerance even for a concentration of 5 g/100 ml (50, 000 mg/ml) (Table 21). *P. fluorescens* was found to be more compatible than *B. subtilis* with fungicides, the MTC for the former being 2500 µg Thiram/ml, 1600 µg mancozeb/ml and 5 g/100 ml for captan and carbendazim and 8000 µg nemacur/ml (Table 21).

Indole acetic acid (IAA) production in liquid medium

IAA production varied with isolates. Highest amount of IAA was produced by the standard strain of *P. fluorescens* (22.6 mg/ml) followed by *B. subtilis* (18.0 mg/ml). The soil isolates produced IAA in the order Pf 01 > Pf 02 > Pf 03 > BS04 (Table 22).

Phosphate solubilization in liquid medium

Quantitative estimation of phosphorus solubilization showed maximum solubilization, 6.0 and 5.6 µg P/ml by *P. fluorescens* and *B. subtilis,* respectively followed by 5.8 (Pf02) and 5.4 µg/ml (BS 02) (Table 23; Fig. 15). The solubilization

Table 21. *In vitro* compatability of *Trichoderma harzianum, T. virens, Pochonia chlamydosporia, Bacillus subtilis* and *Pseudomonas fluorescens* with some common fungicides. MTC- Maximum tolerance concentration; MIC- Maximum inhibition concentration.

	T. harzianum		*T. virens*		*P. chlamydosporia*		*B. subtilis*		*P. fluorescens*	
	MTC*	MIC	MTC	MIC	MTC	MIC	MTC	MIC	MTC	MIC
Carbendazim	60	500	40	405	37.5	250	50, 000	-	50, 000	-
Metalaxyl	1050	2400	1000	2200	100	500	7,000	10,000	10,000	25,000
Captan	160	1000	125	875	75	500	3200	4000	50, 000	-
Mancozeb	225	755	177	625	110	350	600	1000	1600	2000
Thiram	25	150	9	95	5	50	60	100	2500	3000
Nemacur	980	1150	700	1000	250	450	3500	4200	8000	9000

Each value is mean of three replicates. * Concentrations are in (µg/ml).

Table 22. Indole acetic acid (IAA) production by *Bacillus subtilis* and *Pseudomonas fluorescens* strains/isolates in liquid medium supplemented with tryptophan 35 mg/ 100 ml.

Organism	IAA (µg/ml)	Organism	IAA (µg/ml)
B. subtilis (Bs 00)*	18	*P. fluorescens* (Pf 00)*	22.6
Bs 01	10.5	Pf01	20.5
Bs 02	13.0	Pf02	20.0
Bs 03	9.5	Pf03	18.0
Bs 04	16.0	Pf04	15.0
Bs 05	13.0	Pf05	17.0

Each value is mean of three samples; * Standard strains otherwise soil isolates.

Table 23. Phosphorus solubilization by *Bacillus subtilis* and *Pseudomonas fluorescens* strains/ isolates in Pikovskaya's liquid medium.

Bacteria	Phosphorus (µg/ml)	pH	Bacteria	Phosphorus (µg/ml)	pH
B. subtilis (Bs 00)*	5.6	4.7	*P. fluorescens* (Pf 00)*	6.0	4.6
Bs 01	5.3	5.0	Pf01	5.6	4.7
Bs 02	5.4	4.9	Pf02	5.8	4.8
Bs 03	5.0	5.2	Pf03	5.3	5.2
Bs 04	5.0	5.1	Pf04	5.0	5.5
Bs 05	5.3	5.0	Pf05	5.3	5.1

Each value is mean of three samples; * Standard strains otherwise soil isolates.

was coupled with a fall in pH of the medium. The maximum decrease in pH of the medium from 7.0 to 4.6 was recorded with *P. fluorescens* strain/isolates.

Mass culture of biocontrol agents on agricultural wastes

Colonization by *Trichoderma harzianum* was good (90-100%) on most of the media tested, except husk + sand + molasses (15% colonization) (Table 24; Fig. 15).

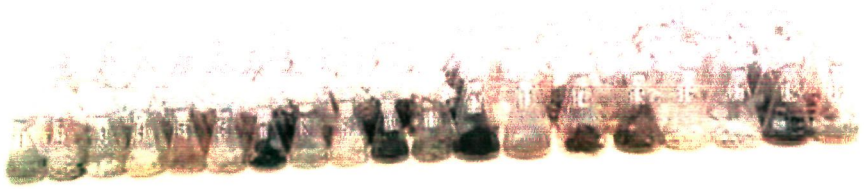


Figure 15. Biocontrol fungi cultured on various materials in flasks

The spore load of the fungus in terms of colony forming units/g media was greatest on sawdust + molasses and bagasse + soil + molasses. On the husk + sand + molasses, the CFU counts were low. Colonization by *T. virens* was 100% on corn meal + sucrose, bagasse + soil + molasses, wheat meal + sucrose and corn cob + sand + molasses. The CFU count was greatest on sawdust + molasses, corn meal + sucrose mixture, bagasse + soil + molasses and wheat meal + sucrose. On husk + sand + molasses and corn cob + molasses, very low CFUs were detected. Colonization by *Pochonia chlamydosporia* was 100% on saw dust + molasses, corn meal + sucrose + mixture and wheat meal + sucrose (Table 19). The CFU counts were greatest on corn meal + sucrose mixture, high but significantly less on oat kernel + sucrose and low on other media. The fungus neither colonized nor produced spores on corn cob + sucrose, corn cob + molasses and corn cob + sand + molasses. Among *Trichoderma* species, *T. harzianum* was the fastest and most efficient colonizer growing on most of the media tested. *P. chlamydosporia* was highly selective, growing on only 8 of the media tested and produced low CFUs. Saw dust + molasses, and bagasse + soil + molasses were the best media for mass culture of the biocontrol agents. CFU counts of the organisms on bagasse + soil + molasses, and saw dust + molasses were comparable with available biopesticides in India. Since India is amajor sugarcane producing country, bagasse, saw dust and molasses are available locally

Table 24. Colonization and sporulation of biocontrol fungi, *Trichoderma harzianum, T. virens* and *Pochonia chlamydosporia* on agricultural materials.

Media	*T. harzianum*		*T. virens*		*P. chlamydosporia*	
	Colonization %	CFU/g ($x 10^4$)	Colonization %	CFU/g ($x 10^4$)	Colonization %	CFU/g ($x 10^4$)
Corn Grain + Sucrose	93	108	80	80	90	60
Corn Meal + Sucrose	98	113	100	106	100	110
Compost + Sucrose	91	102	50	81	-	-
Leaf litter + Sucrose	100	122	33	17	90	65
Wheat Grain + Sucrose	100	103	60	84	20	
Wheat Meal + Sucrose	100	105	100	105	100	55
Oat Kernal + Sucrose	100	108	86	25	50	85
Corn Cob + Sucrose	90	111	15	24	-	-
Corn Cob + Molasses	90	113	3	5	-	-
Corn Cob + Sand + Molasses	100	96	100	95	-	-
Bagasse + Soil + Molasses	100	124	100	106	90	70
Saw Dust + Molasses	100	130	100	110	100	78
Husk + Sand + Molasses	15	2	13	2	50	10

Each value is mean of three replicates.

at low cost. Their use as a media to mass culture of biocontrol fungi will create an opportunity to farmers to produce their own mass culture of organism if a suitable technology is developed and transferred to them.

Selection of strains/isolates for further study

Based on *in vitro* performance of the standard strains and soil isolates of biocontrol agents; the standard strains viz., *Trichoderma harzianum* (Th 00), *T. virens* (Tv 00), *Pochonia chlamydosporia* (Pc 00), *Bacillus subtilis* (Bs 00) and *Pseudomonas fluorescens* (Pf 00) were selected for further study for the reason that they showed faster growth rate, had more antipathogenic activity, solubilized phosphorus with greater efficiency and produced more IAA than other isolates.

Experiment – II

POT TRIAL FOR EVALUATION OF RELATIVE EFFECTIVENESS OF SOME BIOCONTROL FUNGI AND BACTERIA AGAINST WILT, ROOT-KNOT AND WILT DISEASE COMPLEX OF CHICKPEA

Symptoms

Fusarial wilt

Inoculation with the pathogenic fungus, *Fusarium oxysporum* f. sp. *ciceri* (2 g colonized sorghum seeds/kg soil) caused characteristic symptoms of the wilt disease. The first sign of the disease was stunted growth and mild chlorosis that appeared at seedling stage. Some plants at the seedling stage exhibited drooping that led to their mortality. The seedlings which escaped early infection showed chlorosis and stunted growth at one month of age. At the advanced stage of plant growth, the whole plant, branches or twigs became brown and subsequently dried and died (Fig. 16). The severity of wilt on an average was 3.6 on 0-5 scale (Fig. 17). Application of biocontrol agents through soil or seed treatment decreased the severity of wilt symptoms. Greatest decrease in the wilting occurred with seed treatment (67 & 64%) and soil application (67 & 61%) of *T. harzianum* and *T. virens* respectively, followed by *B. subtilis* (57%). Treatment with *P. fluorescens* controlled the wilting by 56% (seed treatment) and 53% (soil application). Application of *P. chlamydosporia* checked the wilt severity by 19% whereas carbendazim treatment by 42-53%. Application of nemacur did not influence the wilting.

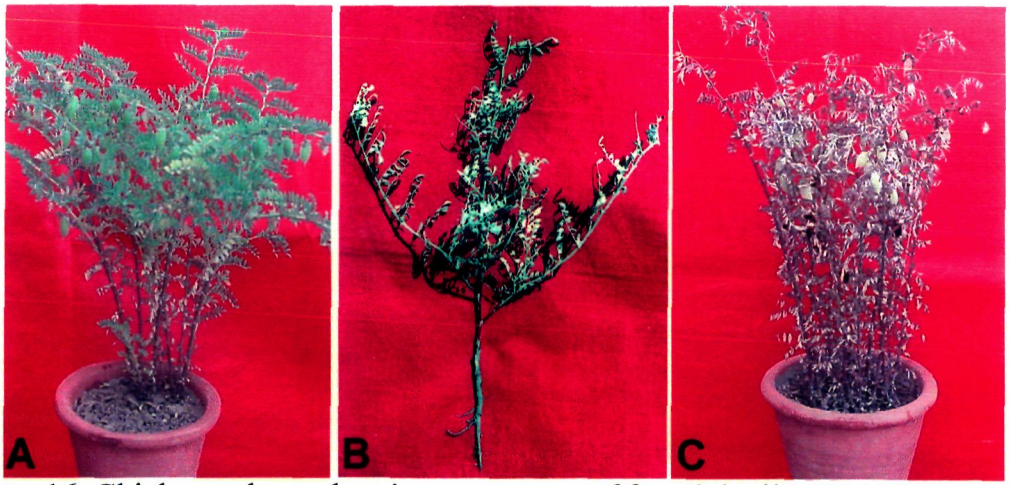


Figure 16. Chickpea plants showing symptoms of fusarial wilt. (A) Uninoculated healthy plant. (B) Browning and wilting of shoot at young and (C) maturing stage.

Figure 17. Effects of seed treatment and soil application of biocontrol agents on the wilt severity (0-5 scale) of chickpea caused by *Fusarium oxysporum* f. sp. *ciceri* alone or with *Meloidogyne incognita.*

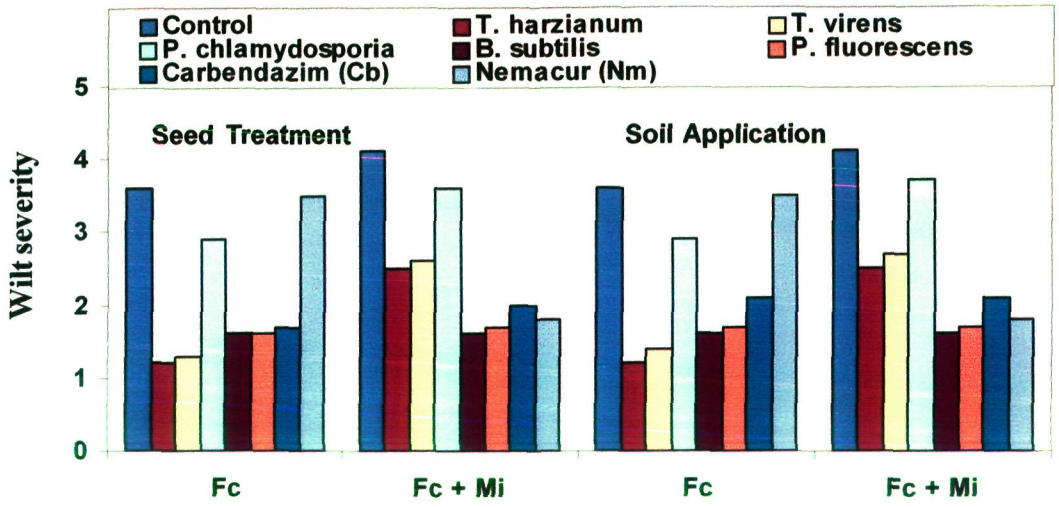
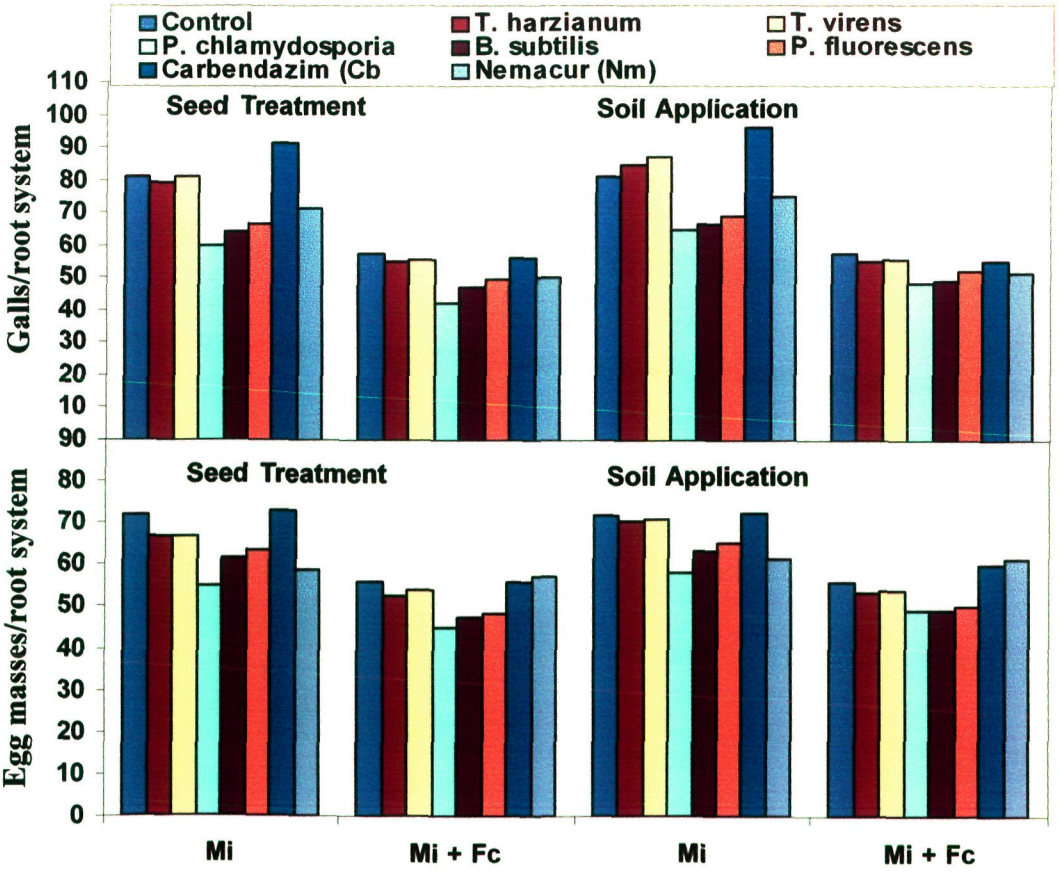


Figure 18. Effect of seed treatment and soil application of biocontrol agents on the root-knot disease and reproduction of *Meloidogyne incognita* alone or with *Fusarium oxysporum* f. sp. *ciceri.*



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

Root-knot symptoms

Chickpea plants inoculated with 2000 J_2 of *Meloidogyne incognita* /pot, exhibited stunted growth and mild chlorosis. On an average 81 galls and 72 eggmasses/root system were recorded (Fig 18 & 19). The applied biocontrol agents suppressed the gall formation and egg mass production but to a varied extent. *Pochonia chlamydosporia* caused the highest suppression in the number of galls (20% decrease by soil application; 26% by seed treatment) and egg mass production (19% decrease by soil application; 24% by seed treatment). Effect of *B. subtilis* were more or less similar to *P. chlamydosporia*. Treatment with *P. fluorescens* also significantly suppressed ($P \leq 0.05$) the galling and egg mass production by 15 and 9%, and 18 and 12% with soil and seed treatment, respectively. Application of nemacur caused 7-12% and 14-19% decrease in the number of galls and egg masses respectively. *Trichoderma* spp. and carbendazim treatments did not influence the nematode galling and egg mass production.

Figure 19. Roots of chickpea showing severe galling caused by *Meloidogyne incognita*

Disease complex

Severity of wilt caused by *F. oxysporum* f. sp. *ciceri* increased significantly ($P \leq 0.05$) in the pots also inoculated with *M. incognita* but gall formation and egg mass production were lower ($P \leq 0.05$) in comparison to the pots with either pathogen alone (Fig. 17). Application of biocontrol agents resulted to decrease in the severity

of wilt and/or root-knot. Greatest decrease in the wilt symptoms of concomitantly inoculated plants was recorded due to seed treatment (61%) or soil application (61%) by *B. subtilis* in comparison to the inoculated control. *P. fluorescens* were next in effectiveness and decreased the wilting by 58%. Application of *T. harzianum* and *T. virens* decreased the wilting by 34-39% over control. The pesticides nemacur and carbendazim however, checked the wilt symptoms by 56-58% and 48-51%, respectively. Gall formation of concomitantly inoculated plants was reduced by 26 and 15% due to seed and soil treatment with *P. chlamydosporia* compared with the control; corresponding values for *B. subtilis* were 18 and 14%. With *P. fluorescens* treatment the galling was 10-13% less than the control. Soil application or seed treatment with nemacur suppressed the gall formation by 12 and 10%. The fungicide and *Trichoderma* spp. did not influence the galling. Similar effects of various treatments were recorded on egg mass production (Fig. 18).

Dry matter production and yield

Chickpea plants applied with *B. subtilis* produced dry matter 45-46% and yield 35-36% greater than those not applied with the bacteria (Table 25). This growth promoting effect was greater with the seed treatment. Application of *P. fluorescens* also improved the dry matter production of chickpea but relatively less than *B. subtilis*.

Infection by the wilt fungus suppressed the dry matter production and yield of chickpea by 18 and 17%, respectively than the control. Application of various treatments compensated the yield loss but to a varying extent. Seed treatment with *B. subtilis* promoted the dry matter production and yield by 76 and 58%, respectively. Corresponding values for soil application were 66 and 54%, respectively. *P. fluorescens* were next in effectiveness inducing 53-75% enhancement in the variables considered. *Trichoderma* spp. also induced significant effect. The plants also gave significantly greater yield with the treatments with carbendazim and nemacur.

Chickpea plants infected with root-knot nematode, *M. incognita* produced dry matter and yield by 12 and 10% less than the control (Table 25). Application of *P. chlamydosporia* significantly promoted the dry matter production and yield of nematode infected plants in comparison to the nematode inoculated control, the effect was 8-9% greater with the seed treatment than soil application. Seed treatment

Table 25. Effect of seed treatment and soil application of biocontrol agents on the dry matter production and yield of chickpea in pots inoculated singly or concomitantly with *Fusarium oxysporum* f. sp. *ciceri*, *Meloidogyne incognita* or not inoculated.

Treatments	Dry shoot weight (g)		Yield/plant (g)	
	Seed Treatment	Soil Application	Seed Treatment	Soil Application
Control (C1)	19.5	19.5	6.9	6.9
T. harzianum (Th)	27.1 (39.0)	27.0 (38.5)	8.0 (15.9)	7.6 (10.1)
T. virens (Tv)	25.8 (32.3)	25.9 (32.8)	8.2 (18.8)	8.1 (17.4)
P. chlamydosporia (Pc)	21.7 (11.3)	21.3 (9.2)	7.6 (10.1)	7.3 (5.8)
B. subtilis (Bs)	28.5 (46.1)	28.3 (45.1)	9.3 (34.8)	9.4 (36.2)
P. fluorescens (Pf)	28.2 (44.6)	28.0 (43.6)	9.4 (36.2)	9.2 (33.3)
Carbendazim (Cb)	22.3 (14.3)	21.9 (12.3)	7.5 (8.7)	7.2 (4.3)
Nemacur (Nm)	22.0 (12.8)	21.3 (9.2)	7.5 (8.7)	7.3 (5.8)
Control (C2)	16.0 (-17.9)	16.0 (-17.9)	5.7 (-17.4)	5.7 (-17.4)
F + *T. harzianum* (Th)	25.1 (56.9)	24.3 (51.9)	8.5 (49.1)	8.2 (43.8)
F + *T. virens* (Tv)	24.2 (51.2)	23.9 (49.4)	8.3 (45.6)	8.1 (42.1)
F + *P. chlamydosporia* (Pc)	17.3 (8.1)	17.0 (6.2)	6.1 (7.0)	6.0 (5.3)
F + *B. subtilis* (Bs)	28.1 (75.6)	26.5 (65.6)	9.0 (57.9)	8.8 (54.4)
F + *P. fluorescens* (Pf)	28.0 (75.0)	27.5 (71.9)	8.9 (56.1)	8.7 (52.6)
F + Carbendazim (Cb)	21.0 (31.2)	21.3 (33.1)	7.0 (22.8)	6.7 (17.5)
F + Nemacur (Nm)	17.2 (7.5)	16.5 (3.1)	6.3 (10.5)	6.0 (5.3)
Control (C3)	17.2 (-11.8)	17.2 (-11.8)	6.2 (-10.1)	6.2 (-10.1)
N + *T. harzianum* (Th)	19.8 (15.1)	19.1 (11.0)	6.9 (11.3)	6.5 (4.8)
N + *T. virens* (Tv)	19.6 (13.9)	18.9 (9.9)	6.9 (11.3)	6.1 (1.6)
N + *P. chlamydosporia* (Pc)	23.8 (38.4)	22.3 (29.6)	8.1 (30.6)	8.0 (29.0)
N + *B. subtilis* (Bs)	23.5 (36.6)	22.5 (30.8)	8.8 (41.9)	8.5 (37.1)
N + *P. fluorescens* (Pf)	23.2 (34.9)	22.1 (28.5)	8.6 (38.7)	8.3 (33.9)
N + Carbendazim (Cb)	18.0 (4.6)	17.5 (1.7)	6.4 (3.2)	6.3 (1.6)
N + Nemacur (Nm)	20.3 (18.0)	20.0 (16.3)	7.4 (19.3)	7.2 (16.1)
Control (C4)	12.1 (-37.9)	12.1 (-37.9)	4.4 (-36.2)	4.4 (-36.2)
F + N + *T. harzianum* (Th)	16.2 (33.9)	16.0 (32.2)	5.2 (18.2)	5.0 (13.6)
F + N + *T. virens* (Tv)	16.1 (33.0)	16.3 (34.7)	5.1 (15.9)	5.3 (20.4)
F + N + *P. chlamydosporia* Pc	15.0 (24.0)	14.3 (18.2)	5.1 (15.9)	4.8 (9.1)

Continued.....

Continued..... Table 25

F + N + *B. subtilis* (Bs)	19.4 (60.3)	18.5 (52.9)	6.5 (47.7)	6.2 (40.9)
F + N + *P. fluorescens* (Pf)	19.2 (58.7)	18.3 (51.2)	6.4 (45.5)	6.0 (36.4)
F + N + Carbendazim (Cb)	15.9 (31.4)	15.7 (29.7)	5.0 (13.6)	4.8 (9.1)
F + N + Nemacur (Nm)	17.0 (40.5)	16.3 (34.7)	5.3 (20.4)	5.0 (13.6)
LSD ($P \leq 0.05$)	0.12	0.05	0.10	0.06
F-value $P \leq 0.05$				
Control agents (df = 7)	1613.12*	7129.90*	231.88*	1244.99*
Pathogens (df = 1)	376.34	1121.54	341.13	1549.65*
Interaction (df = 7)	44.53*	144.23*	NS	36.14*

Values in parenthesis are percent increase (+ ve) or decrease (- ve) over respective control (Plants not inoculated with either pathogen but applied with biocontrol agents were compared with Uninoculated Control C1; wilt fungus inoculated plants applied with biocontrol agents were compared with fungus inoculated control C2; nematode inoculated plants applied with biocontrol agents were compared with nematode inoculated control C3; concomitantly inoculated plants applied with biocontrol agents were compared with concomitantly inoculated control C4). * Significantly different from the control at $P \leq 0.05$; NS- Not significant at $P \leq 0.05$.

with *B. subtilis* or *P. fluorescens* increased the yield by 34-42% over control. The yield of chickpea was also significantly improved due to application with nemacur.

Concomitant inoculations with wilt fungus and root-knot nematode greatly suppressed the dry matter production (38%) and yield (36%) of chickpea over uninoculated control (Table 25). All treatments applied significantly improved the considered variables of infected plants in comparison to the concomitantly inoculated control. Greatest enhancement in the dry matter and yield of infected plants was recorded with seed treatment (60 and 48%) or soil application (53 and 41%) by *B. subtilis* compared to the concomitantly inoculated control. Next in effectiveness was *P. fluorescens* that gave 36-45% increase in the yield. Treatment with *T. harzianum* however, improved the dry matter and yield by 32-34% & 14-18%, respectively. The pesticide treatment, nemacur and carbendazim significantly ($P \leq 0.05$) increased the dry matter production and yield over the control.

Root nodulation

The nodulation induced by the *Rhizobium ciceri* in chickpea roots was quite good (Fig. 20) and it further increased in the presence of all biocontrol agents used, being greater with *B. subtilis*. Infection by *F. oxysporum* f. sp. *ciceri* and *M. incognita* singly or concomitantly resulted to a decrease in the number of functional and total nodules per root system in comparison to the control (Fig. 21). Decrease in the nodulation by concomitant inoculation of the pathogens was significantly greater than their individual effects ($P \leq 0.05$). Application of various treatments checked the suppressive effect of the pathogens on root nodulation. Application of *B. subtilis* through soil or seed treatment increased the number of functional nodules by 31 and 30%, respectively, in the plants infected with the wilt fungus in comparison to the respective controls (Fig. 21).

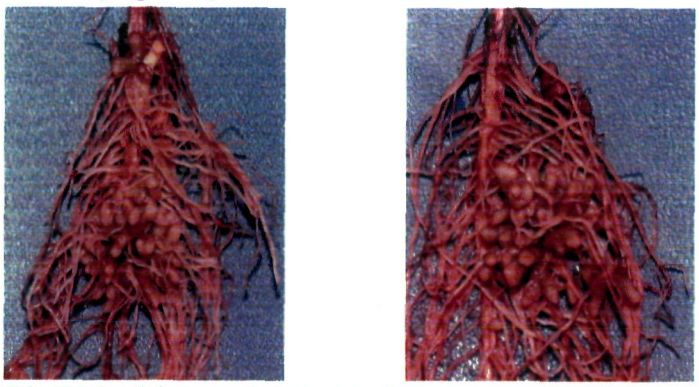
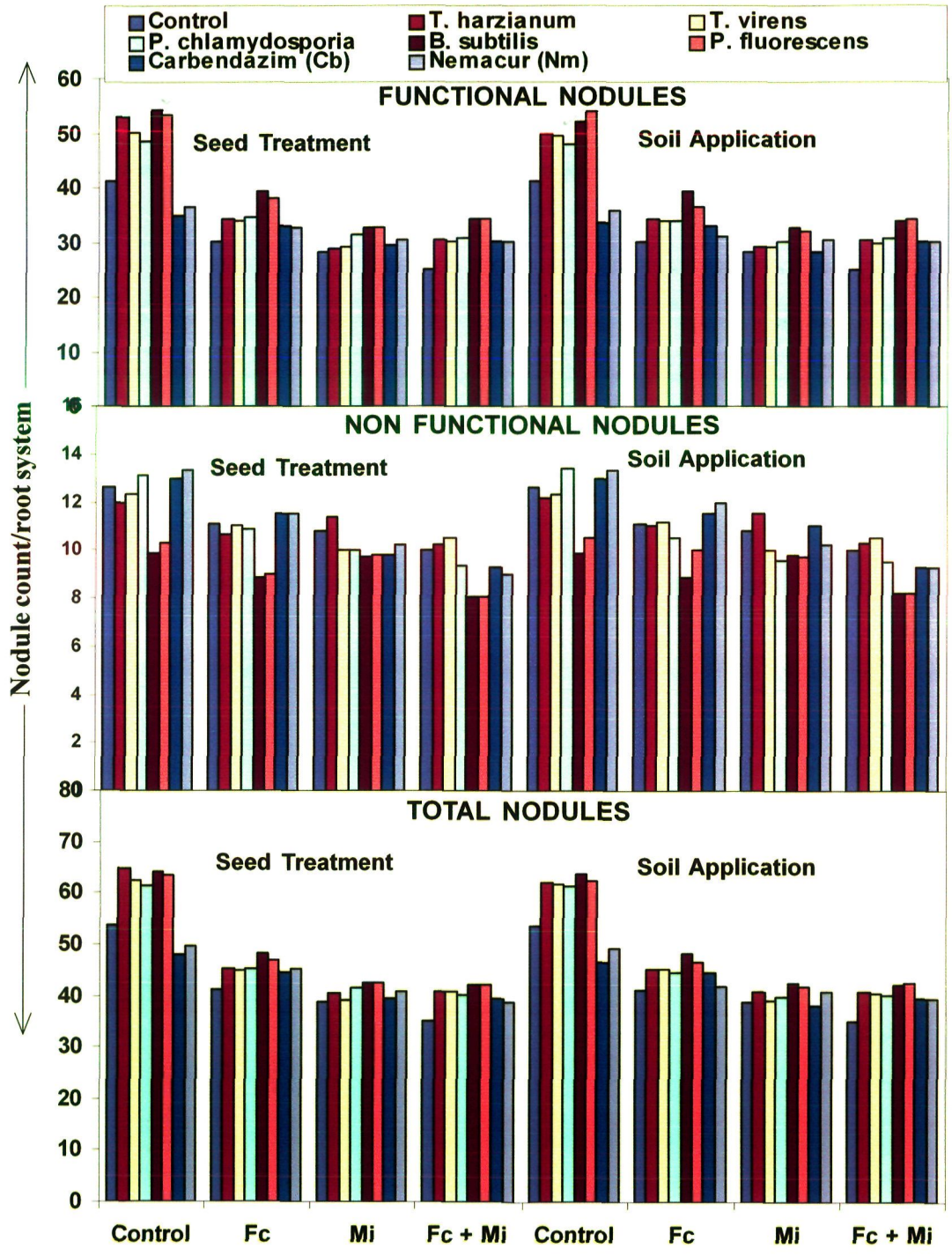


Figure 20. Rhizobial nodules on the roots of chickpea

Figure 21. Effects of seed treatment and soil application of biocontrol agents on the nodulation of chickpea inoculated with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita* or not inoculated.



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

Next in nodule promotion was *P. fluorescens, P. chlamydosporia, T. harzianum, T. virens,* carbendazim and nemacur.

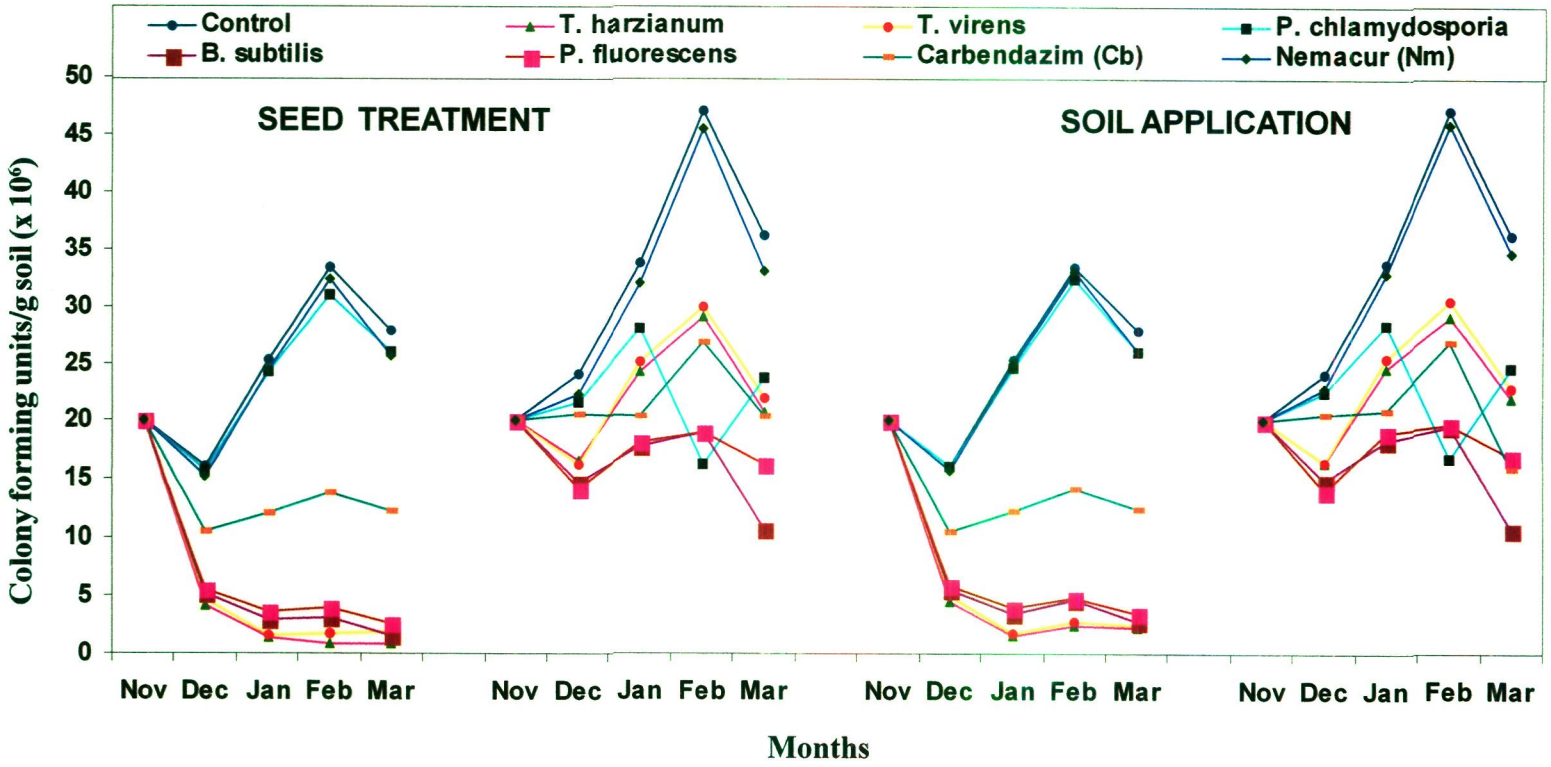
Root nodulation in nematode infected plants also improved with most of the treatments applied. Greatest promotion in the functional nodules of nematode infected plants occurred with *B. subtilis* (16-17%), followed by *P. fluorescens* (14-16%) and *P. chlamydosporia* (8-12%) in comparison to the nematode inoculated control (Fig. 21). Nemacur application resulted to 8-9% increase in the functional nodules (Fig. 21). Almost similar effect of various treatments was recorded on the nodulation of concomitantly inoculated plants and the order of nodule promotion was *B. subtilis* > *P. fluorescens* > *P. chlamydosporia*> *T. harzianum* > *T. virens* (Fig. 21). The order in the decrease of nonfunctional nodules was *B. subtilis* = *P. fluorescens* > Nemacur > carbendazim > *P. chlamydosporia. Trichoderma* spp. increased non functional nodules.

Soil population

Wilt fungus, *Fusarium oxysporum* f. sp. *ciceri*

Soil population of the wilt fungus, *F. oxysporum* f. sp. *ciceri* gradually increased during four month period of experiment reaching to a peak of 33.4 x 10^6 CFUs/g soil in February in comparison to the planting population of 20 x 10^6 CFUs (Fig.22). Various treatments, however, caused decrease in the pathogen population but to a varying extent (Fig. 22). Greatest decrease was recorded with the soil or seed treatment of *T. harzianum* (72-97%) followed by *B. subtilis* (66-95%) in comparison to planting population as well as respective month control (Fig. 22). Next in effectiveness was *T. virens* followed by *P. fluorescens* through seed treatment (Fig. 22). Seed treatment by carbendazim decreased the pathogen population by 30-47% over planting population. In the presence of root-knot nematode, *M. incognita*, soil population of the wilt fungus was significantly greater ($P \leq 0.05$) with an increase of 30-49% in comparison to the monthly population in the absence of nematode (Fig. 22). Population of the fungus in concomitantly inoculated plants also decreased due to application of various treatments (Fig. 22). Seed treatment with *B. subtilis* caused greatest decrease (5-46%) in the pathogen population followed by *P. fluorescens* (5-29%) as compared to preplant population (Fig. 22). *T. harzianum* and *T. virens* decreased the pathogen population in December by 17 and 19% as compared to preplant population.

Figure 22. Effects of seed treatment and soil application with biocontrol agents on the soil population of *Fusarium oxysporum* f. sp. *ciceri* in the presence and absence of *Meloidogyne incognita.*



Root-knot nematode, *Meloidogyne incognita*

Soil population of *M. incognita* was gradually increased with the progress of time from December onwards and reached its peak at harvest that was 96.8% greater than planting population of 2000 J_2/kg soil (Fig. 23). In the presence of wilt fungus, nematode population, however, was significantly less than the respective month controls ($P \leq 0.05$). Seed treatment with *P. chlamydosporia* significantly suppressed the population of nematode by 15-55% followed by *B. subtilis* 11-53% (Fig. 23). Next in effectiveness were *P. fluorescens* and nemacur (11-52%) as compared to preplant treatment. Soil application of various treatments was relatively less effective than seed treatment in suppressing the nematode population in the absence of wilt fungus (Fig. 23). In the presence of wilt fungus, nematode population increased over time, the increase was, however, significantly less than the nematode alone (Fig. 23). Seed treatment with the biocontrol agents or pesticides decreased the nematode population and order of decrease was more or less similar to the treatments where nematode was applied alone. Soil application of various treatments was more or less equally effective in suppressing the nematode population in the presence of wilt fungus.

Biocontrol agents

***Trichoderma* species**

Soil population of *T. harzianum* significantly increased during the experimental period (Fig. 24). Soils infested with wilt fungus alone or together with nematode, population of *Trichoderma* spp. increased during all months of sampling being greatest in March in comparison to planting population. The percent increase in *T. harzianum* was relatively greater than *T. virens*. In nematode inoculated pots, increase in *T. harzianum* population was significant in comparison to planting populatioonly (Fig. 24).

Pochonia chlamydosporia

Soil population of *P. chlamydosporia* applied through seed treatment or soil application in pots not inoculated with either pathogens was marginally increased in comparison to the planting population, the population also decreased in the pots inoculated with wilt fungus (Fig. 24). With the treatments of nematode alone or together with wilt fungus, soil population of *P. chlamydosporia* significantly

increased during all four months in comparison to planting population or respective month control (Fig. 24).

Bacillus subtilis* and *Pseudomonas fluorescens

Rhizosphere population of *B. subtilis* and *P. fluorescens* significantly increased during all four months of sampling in comparison to planting population (Fig. 24). The population of the bacterium further increased in the presence of pathogens in comparison to respective month controls. Greatest increase in the population was recorded in concomitantly inoculated pots followed by the inoculation with either pathogen in comparison to respective month populations of *B. subtilis* and *P. fluorescens* in the absence of pathogens (Fig. 24). Soil population of *B. subtilis* and *P. fluorescens* increased by 5-55% and 5-57%, respectively, during crop growth from December to March (Fig. 24). Response of *P. fluorescens* and *B. subtilis* population when applied in soil was almost identical to seed treatment (Fig. 24).

Figure 23. Effects of seed treatment and soil application with biocontrol agents on the soil population of *Meloidogyne incognita* in the presence and absence of *Fusarium oxysporum* f. sp. *ciceri.*

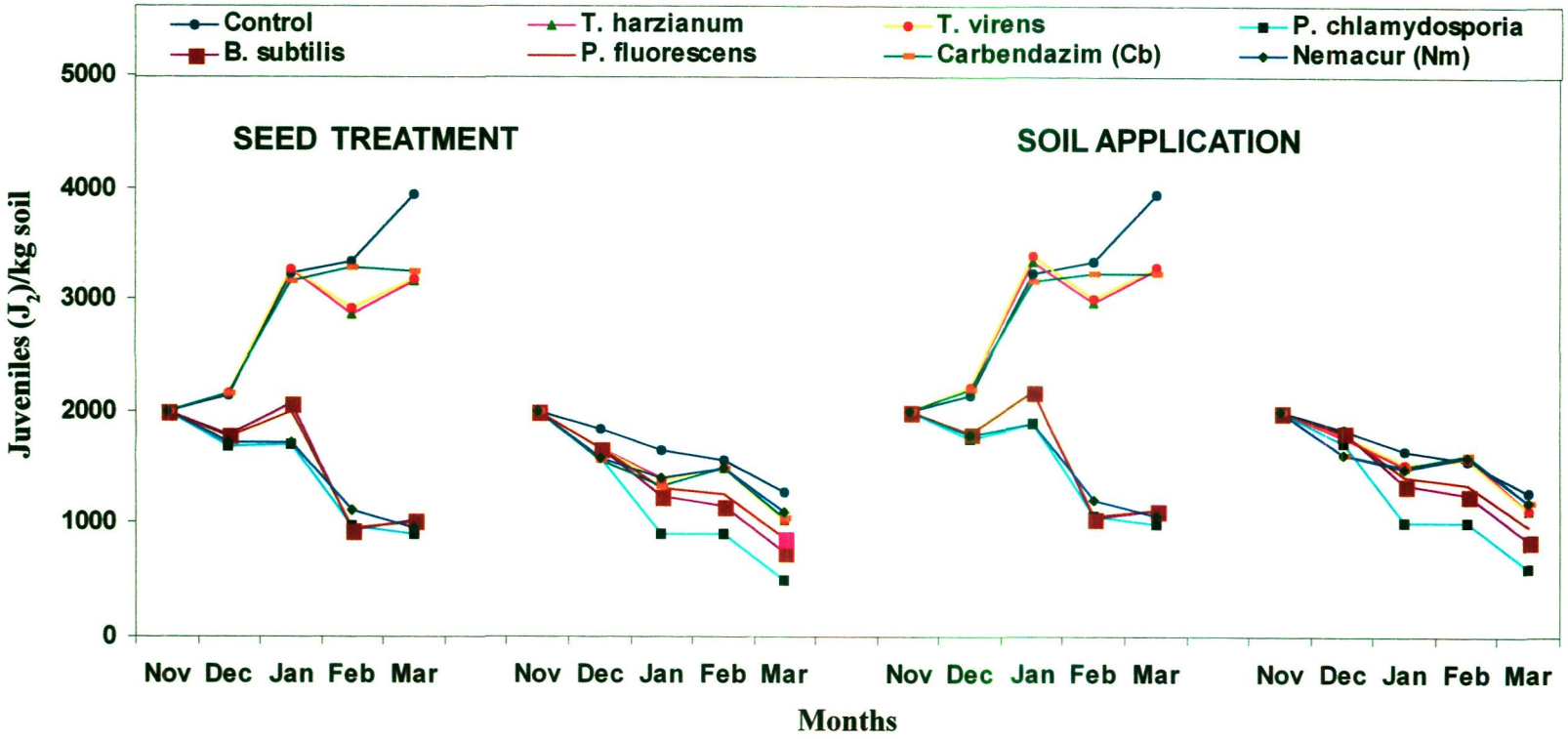
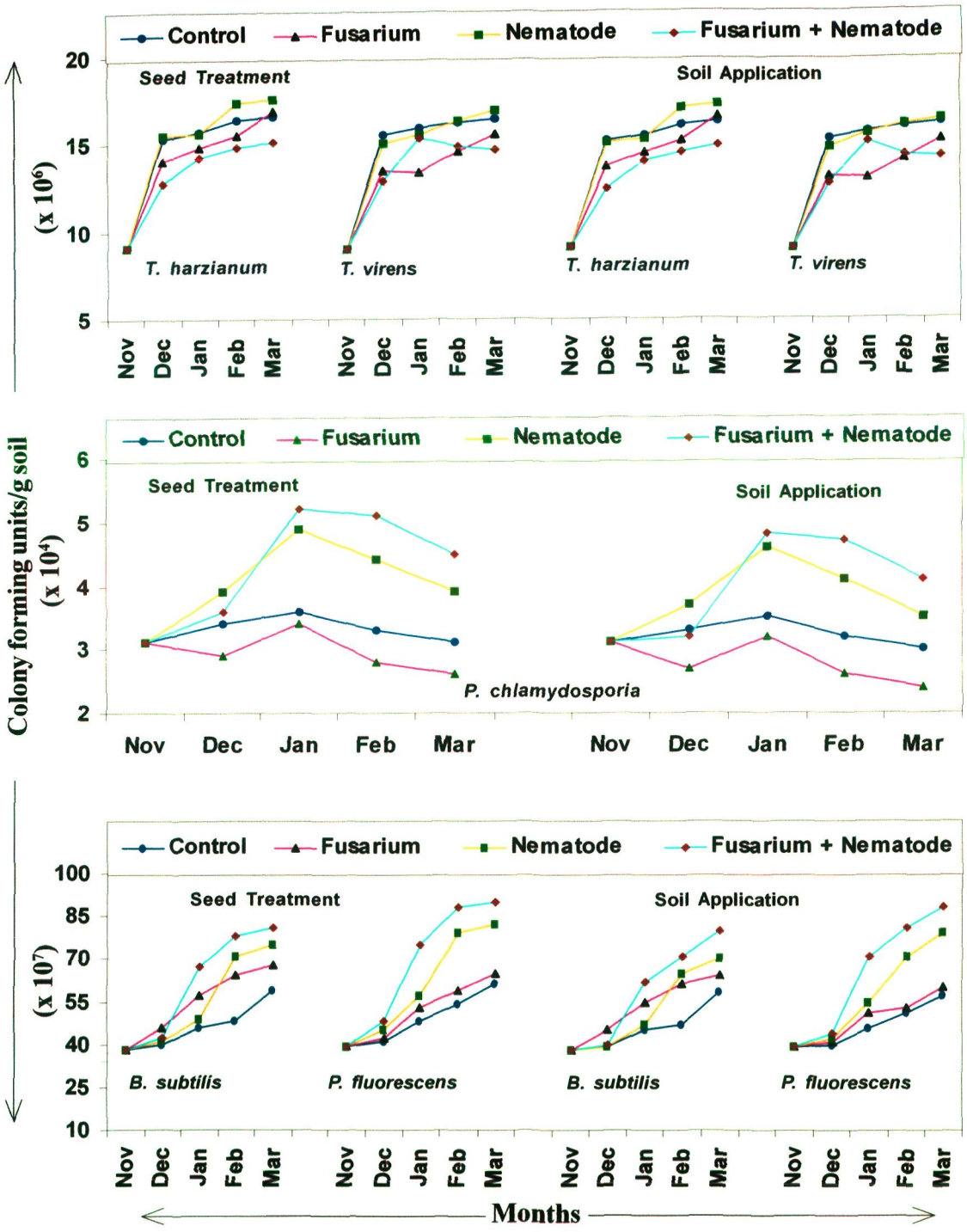


Figure 24. Rhizosphere population of biocontrol agents in relation to single or concomitant inoculations with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.



Experiment III

FIELD TRIAL TO ASCERTAIN THE EFFECTIVENESS OF SELECTED MICROORGANISMS AGAINST FUSARIUM WILT, ROOT-KNOT AND WILT DISEASE COMPLEX OF CHICKPEA

Based on the performance of the biocontrol fungi and bacteria examined in the previous pot culture experiment, three microorganisms viz., *T. harzianum, P. chlamydosporia* and *B. subtilis* were selected for field trials against *F. oxysporum* f. sp. *ciceri* and *M. incognita* because they were found relatively more effective against the target pathogens . The trial was conducted in microplots of 4x2 m size (Fig. 25). Effects of the microorganisms were compared with efficacious pesticides namely carbendazim and nemacur. The microorganisms were cultured on baggase-soil-molasses (BSM) and were applied to seeds (5g//kg seed) and soil (40g/ microplot).

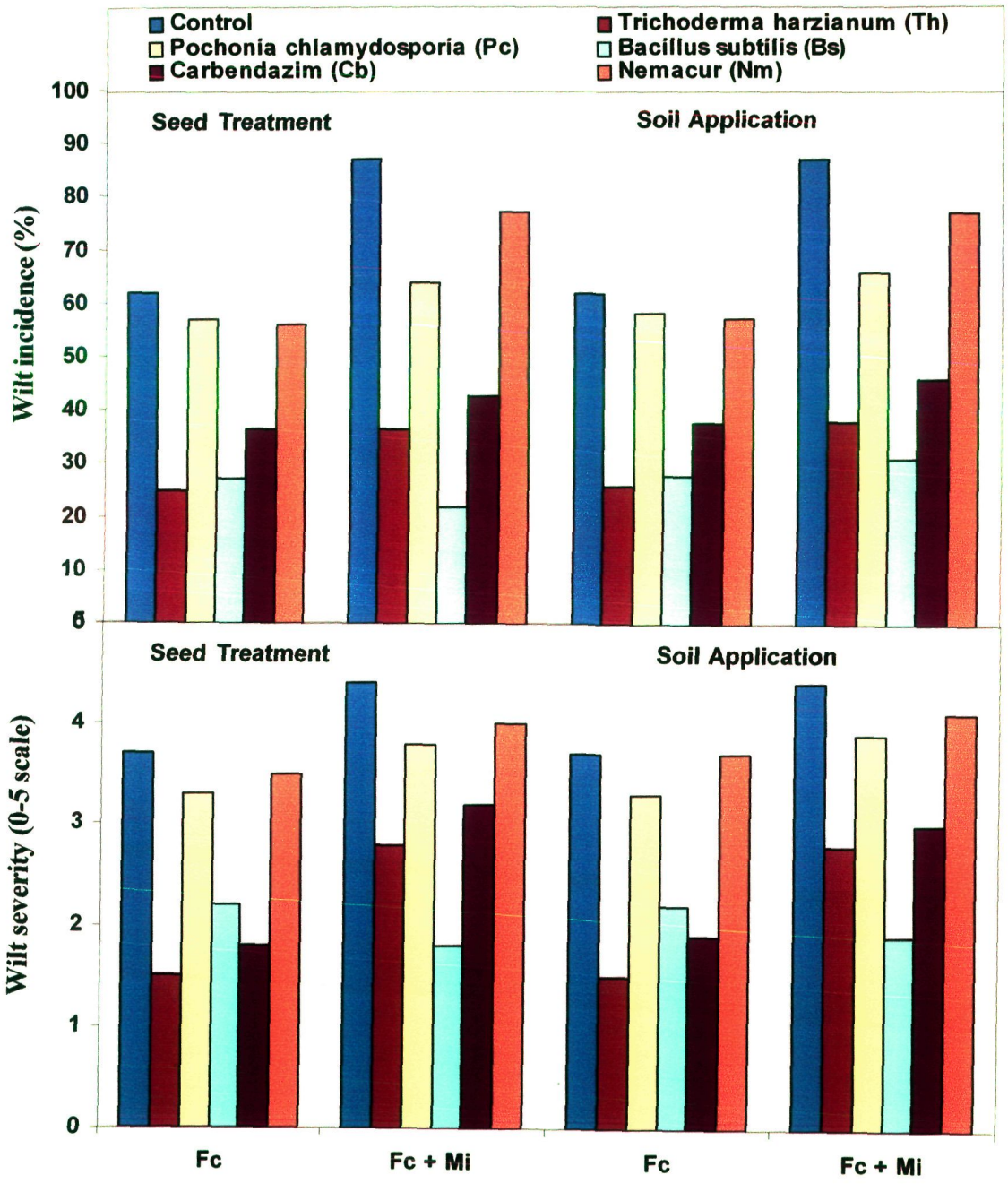
Figure 25. An aerial view of experimental plots

Symptoms

Fusarial wilt

In the plots inoculated with *F. oxysporum* f. sp. *ciceri* the wilt incidence was 62% on chickpea cv. BG-256 (Fig. 26). Application of biocontrol agents or pesticides influenced the disease incidence. Seed treatment with *B. subtilis* or *T. harzianum* brought down the wilt incidence to 25-27% (Fig. 26). In the plots where carbendazim was applied (seed treatment), the wilt incidence was 36%. Application of *P.*

Figure 26. Effects of seed treatment and soil application of biocontrol agents on the incidence and severity of wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceri* in the presence and absence of *Meloidogyne incognita*.



Fc- ***Fusarium oxysporum*** **f. sp.** ***ciceri*****; Mi-** ***Meloidogyne incognita***

chlamydosporia and nemacur decreased the wilt incidence by 8 and 9%, respectively. The severity of wilt due to the pathogen was 73%. Application of biocontrol agents except *P. chlamydosporia* decreased the severity of wilt to a varied extent. Lowest severity (31%) was recorded due to seed treatment with *T. harzianum* followed by 40% with *B. subtilis*, and 36% with carbendazim. Response of wilt disease to soil application of biocontrol agents was more or less similar to that recorded with the seed treatment.

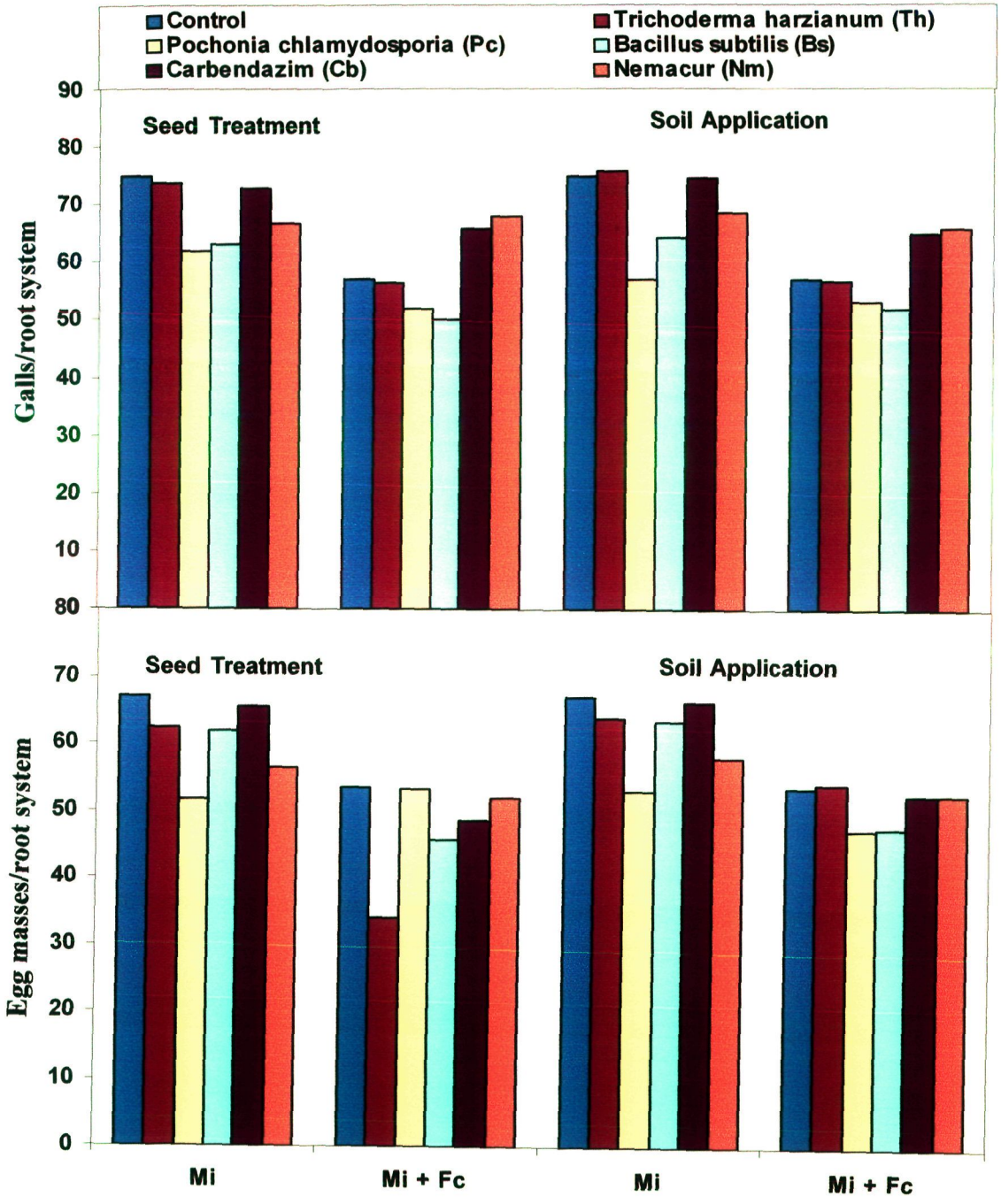
Root-knot disease

Characteristic galls developed on the roots of plants grown in the plots inoculated with the juveniles of *M. incognita* (2000J_2/kg soil) (Fig. 27). On average, 75 galls and 68 egg masses/root system were formed. Application of biocontrol agents suppressed the gall formation to a varied extent (Fig. 27). Seed treatment with *P. chlamydosporia* resulted to 18% decrease in the number of galls, followed by 16% by *B. subtilis* in comparison to the control. The gall formation decreased by 11% due to application of nemacur. Application of *T. harzianum* and carbendazim do not show significant effect.

Disease complex

Plants grown in the plots inoculated with *F. oxysporum* f. sp. *ciceri* and *M. incognita* concomitantly exhibited severe stunted growth with chlorotic, wilted and dried leaves. Wilt symptoms in terms of incidence and severity were significantly greater compared to the wilting recorded in the plots inoculated with *F. oxysporum* f. sp. *ciceri* alone. In the plots inoculated with root-knot nematode, *M. incognita* the wilt incidence and severity increased by 41% and 19%, respectively as compared to the absence of nematode (Fig. 26). The wilt incidence and severity of concomitantly inoculated plants considerably decreased due to various treatments. Greatest decrease was recorded by the seed treatment with *B. subtilis* (75 and 59%) followed by *T. harzianum* (58 and 36%). Carbendazim treatment decreased the wilt incidence and severity by 51 and 32%, respectively. The galling and reproduction (egg mass production) of the nematode were adversely affected with 24% decrease in the number of galls/root system. Application of *P. chlamydosporia* and *B. subtilis* caused a significant ($P \leq 0.05$) decrease in the gall formation that ranged 9-16% (Fig. 27). The reproduction (egg mass production) of the nematode showed almost identical

Figure 27. Effects of seed treatment and soil application of biocontrol agents on the galling caused by *Meloidogyne incognita* in chickpea in the presence and absence of *Fusarium oxysporum* f. sp. *ciceri*.



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

response to various treatments. Overall effect of soil application was similar to seed treatments but it was 2-5% less effective (Fig. 27).

Plant growth and yield

Seed treatment with *T. harzianum* and *B. subtilis* significantly ($P \leq 0.05$) increased the dry weight of chickpea plants (Table 26). Greatest increase with a single biocontrol agent was recorded with *B. subtilis* (39%) in comparison to uninoculated control. Inoculation with *Fusarium oxysporum* f. sp. *ciceri* caused 20% decrease in the plant dry weight (Table 21). Application of the biocontrol agents except *P. chlamydosporia* decreased the suppressive effect of the wilt fungus leading to significant increase in the dry weight of chickpea plants. Application of *B. subtilis* and *T. harzianum* increased the dry matter production by 33 and 56%, respectively compared to wilt fungus inoculated control. Seed treatment with carbendazim resulted to 22% increase in the dry weight of fungus inoculated plants. Infection by root-knot nematode, *M. incognita* caused significant decrease (14%) in the dry weight than the uninoculated control (Table 21). Treatments with the biocontrol agents (except *T. harzianum*) checked the damage caused by nematode ($P \leq 0.05$). *P. chlamydosporia* and *B. subtilis* increase the dry weight of nematode infected plants by 25 and 31%, respectively. Nematicide (nemacur) application increased the dry weight of nematode inoculated plants by 14%.

Concomitant inoculations with *M. incognita* and *F. oxysporum* f. sp. *ciceri* decreased the dry weight of chickpea by 42% compared to uninoculated control (Table 21). This decrease was significantly greater than the sum of reductions caused by the fungus and nematode separately. Treatments with the biocontrol agents considerably decreased the suppressive effect of the pathogens. Applications of *B. subtilis* enhanced the dry weight by 55% whereas the increase due to nemacur and carbendazim was 30 and 28%, respectively.

Yield of chickpea in terms of weight of grains per plant was significantly ($P \leq 0.05$) promoted with the application of biocontrol agents (Table 26). Greatest increase in the yield was recorded with *B. subtilis* (36%). Infection by the wilt fungus decreased the yield of chickpea by 18% compared to the control. The suppresive effect of the wilt fungus however, significantly decreased with the application of various treatments. Seed treatment with *T. harzianum, B. subtilis* and carbendazim enhanced the yield of chickpea by 44, 52, and 20%, respectively,

Table 26. Effects of seed treatment and soil application of biocontrol agents on the dry matter production and yield of chickpea in plots infested singly or concomitantly with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.

Treatment	Shoot dry weight (g)		Yield per plant (g)	
	Seed Treatment	Soil Application	Seed Treatment	Soil Application
Control (C1)	21.9	21.9	7.2	7.2
T. harzianum (Th)	25.9 (18.2)	24.6 (12.3)	8.1 (12.5)	7.7 (6.9)
P. chlamydosporia (Pc)	23.2 (5.9)	22.1 (0.9)	7.6 (5.6)	7.3 (1.4)
B. subtilis (Bs)	30.5 (39.3)	29.0 (32.4)	9.8 (36.1)	9.3 (29.2)
Carbendazim (Cb)	23.7 (8.3)	22.2 (1.4)	7.6 (5.6)	7.0 (-2.8)
Nemacur (Nm)	24.0 (9.5)	22.4 (2.3)	7.5 (4.2)	7.4 (2.8)
Control (C2)	17.5 (-20.1)	17.5 (-20.1)	5.9 (-18.0)	5.9 (-18.0)
T. harzianum + F	23.2 (32.6)	21.9 (25.1)	8.5 (44.0)	7.2 (22.0)
P. chlamydosporia + F	18.4 (5.1)	17.9 (2.3)	6.1 (3.4)	5.9 (0.0)
B. subtilis + F	27.3 (56.0)	25.9 (48.0)	9.0 (52.5)	8.4 (42.4)
Carbendazim + F	21.3 (21.7)	20.0 (14.3)	7.1 (20.3)	6.6 (11.9)
Nemacur + F	18.3 (4.5)	18.1 (3.4)	6.4 (8.5)	6.1 (3.4)
Control (C3)	18.9 (-13.7)	18.9 (-13.7)	6.1 (-15.3)	6.1 (-15.3)
T. harzianum + N	20.1 (6.3)	19.2 (1.6)	6.8 (11.5)	6.3 (3.3)
P. chlamydosporia + N	23.7 (25.4)	22.3 (18.0)	7.8 (27.9)	7.3 (19.7)
B. subtilis + N	24.8 (31.2)	23.4 (23.8)	8.2 (34.4)	7.6 (24.6)
Carbendazim + N	20.2 (6.9)	19.7 (4.2)	6.7 (9.8)	6.5 (6.6)
Nemacur + N	21.5 (13.7)	19.9 (5.3)	7.2 (18.0)	6.6 (8.2)
Control (C4)	12.6 (-42.5)	12.6 (-42.5)	3.8 (-47.2)	3.8 (-47.2)
Th+ FN	14.8 (17.5)	13.6 (7.9)	4.5 (18.4)	4.1 (7.9)

Continued.....

Continued..... Table 26

Pc + FN	14.3 (13.5)	13.2 (4.8)	4.4 (15.8)	3.8 (0.0)
Bs + FN	19.6 (55.5)	18.1 (43.6)	5.7 (50.0)	5.3 (39.5)
Cb + FN	16.1 (27.8)	16.0 (27.0)	4.6 (21.0)	4.4 (15.8)
Nm + FN	16.4 (30.1)	15.2 (20.6)	4.8 (26.3)	4.3 (13.1)
LSD ($P \leq 0.05$)	0.38	0.27	0.14	0.08
F-value $P \leq 0.05$				
Control agents (df = 5)	374.57*	273.49*	125.3*	274.84*
Pathogens (df = 1)	86.30*	110.42*	206.35*	506.53*
Interaction (df = 5)	NS	NS	NS	NS

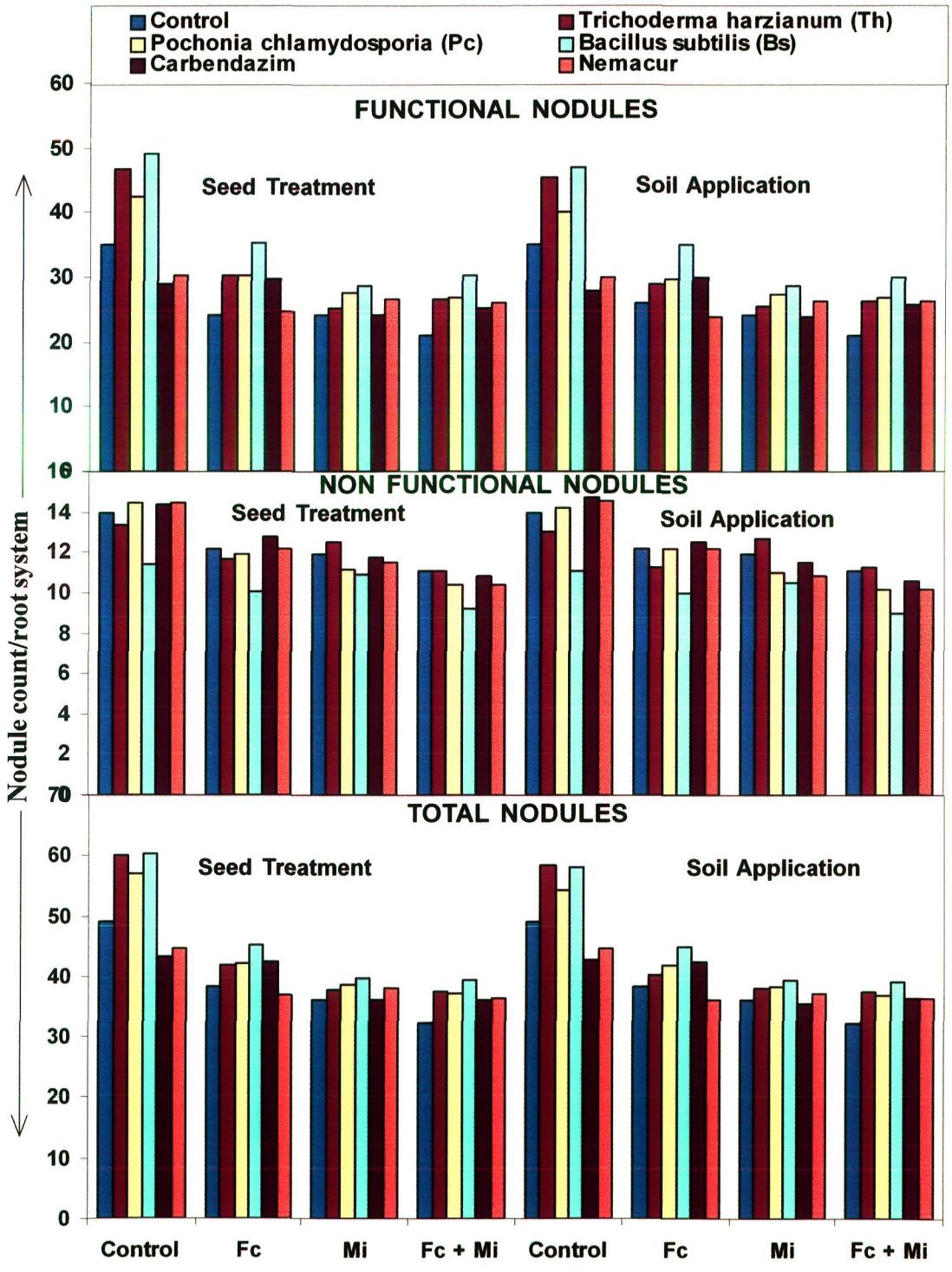
Values in parenthesis are percent increase (+ve) or decrease (-ve) over respective control (Plants not inoculated with either pathogen but applied with biocontrol agents were compared with Uninoculated Control C1; wilt fungus inoculated plants applied with biocontrol agents were compared with fungus inoculated control C2; nematode inoculated plants applied with biocontrol agents were compared with nematode inoculated control C3; concomitantly inoculated plants applied with biocontrol agents were compared with concomitantly inoculated control C4).* Significantly different from the control at $P \leq 0.05$; NS- Not significant at $P \leq 0.05$.

compared to the inoculated control. Root-knot infection caused 15% decline in the yield of chickpea compared to uninoculated control (Table 26). Application of the biocontrol agents singly induced significant increase in the yield of nematode inoculated plants, being greatest with *B. subtilis* (34%), followed by *P. chlamydosporia* (28%) and *T. harzianum* (11%). Application of nemacur resulted to a 18% increase in the yield of chickpea in comparison to nematode inoculated control. Joint inoculation with *F. oxysporum* f. sp. *ciceri* and *M. incognita* drastically suppressed the yield of chickpea (Table 26). The decrease was 17% greater than the sum of the reductions caused by the fungus and nematode separately. *T. harzianum* or *P. chlamydosporia* promoted the yield of infected plants by 16-18%. Application of *B. subtilis* induced a remarkable enhancement i.e., 50% in comparison to concomitantly inoculated control. Effect of soil application of the biocontrol agents on the yield of chickpea plants infected with wilt fungus and/or root-knot nematode was more or less similar seed treatment.

Root nodulation

Nodule formation on the roots of chickpea was quite good (Fig. 28) and on average 49 nodules were formed, out of which 71% were functional and 29% non-functional (Fig. 25). The nodulation was synergised due to the treatments with biocontrol agents. The seed treatment with *B. subtilis*, *T. harzianum*, and *P. chlamydosporia* resulted to 40, 33 and 21% increase, respectively, in the number of functional nodules on chickpea roots over control (Fig. 28). Application of carbendazim or nemacur caused significant decline in the number of functional nodules (14-17%). Infection by *F. oxysporum* f. sp. *ciceri* and *M. incognita* singly or concomitantly significantly suppressed the nodulation ($P \leq 0.05$). Inhibition in the nodulation due to pathogens was greater with concomitant inoculations. Seed treatment with the biocontrol agents suppressed the negative effect of pathogens on functional nodules. Treatment of *B. subtilis* induced greater increase in nodulation on the plants inoculated with the pathogenic fungi and/or nematode (Fig. 28). Number of non-functional nodules decreased invariably with those treatments where number of functional nodules was increased. Carbendazim and nemacur application promoted the number of functional nodules in fungus, nematode and concomitantly infected plants, respectively, but the increase was much less than the effect of *B. subtilis*. Effects of soil application of biocontrol agents was more or less similar to the seed treatments.

Figure 28. Effects of seed treatment and soil application of biocontrol agents on the root nodulation in chickpea grown in the plots infested singly or concomitantly with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

Rhizosphere population of pathogens

Fusarium oxysporum f. sp. *ciceri*

Rhizosphere population of wilt fungus decreased significantly during December (18%) but increased 27-34% with the progress of season in comparison to pre-plant population in November ($P \leq 0.05$) (Fig. 29). Seed treatment with the biocontrol agents or fungicide considerably decreased the population except *P.chlamydosporia* and nemacur. Application of *T. harzianum* or *B. subtilis* caused drastic decrease in the rhizosphere population of *F. oxysporum* f. sp. *ciceris* in comparison to monthly or pre plant control. The decrease by *T. harzianum* was significantly ($P \leq 0.05$) greater than the *B. subtilis*. Seed treatment with carbendazim decreased the pathogen population by 30-54% over planting population. In the presence of root-knot nematode, the population of the wilt fungus was significantly greater than the fungus alone ($P \leq 0.05$). Application of the three biocontrol agents significantly decreased the rhizosphere population in comparison to the control, being greatest with *B. subtilis* (35-67%), followed by *T. harzianum* (29-43%) and *P. chlamydosporia* (10-33%) in comparison to month control. Both pesticides caused significant ($P \leq 0.05$) decrease in the population of *F. oxysporum* f. sp. *ciceri*. Effect of soil application of biocontrol agents on the rhizosphere population was more or less similar to seed treatment (Fig. 29).

Meloidogyne incognita

Soil population of root-knot nematode, *M. incognita* monitored monthly increased gradually and periodically during December to March in comparison to pre-plant population (Fig. 30). The increase was significant in all the months except December. Various treatments influenced the nematode population. Seed treatment with *P. chlamydosporia* or *B. subtilis* significantly decreased the nematode population during all months of sampling by 15-69% and 10-69%, over respective month control. A decrease of 18-83% in the population of *Meloidogyne incognita* was recorded in the plots applied with the nemacur. In the plots, where root-knot nematode and wilt fungus were inoculated concomitantly, the nematode population decreased by 9-64% in comparison to nematode alone. Application of all biocontrol agents significantly ($P \leq 0.05$) decreased the nematode population, the decrease being greater than the nematode alone treatments. Carbendazim treatment decreased the

Figure 29. Effects of seed treatment and soil application with bioagents on the soil population of *Fusarium oxysporum* f. sp. *ciceri* in the presence and absence of *Meloidogyne incognita.*

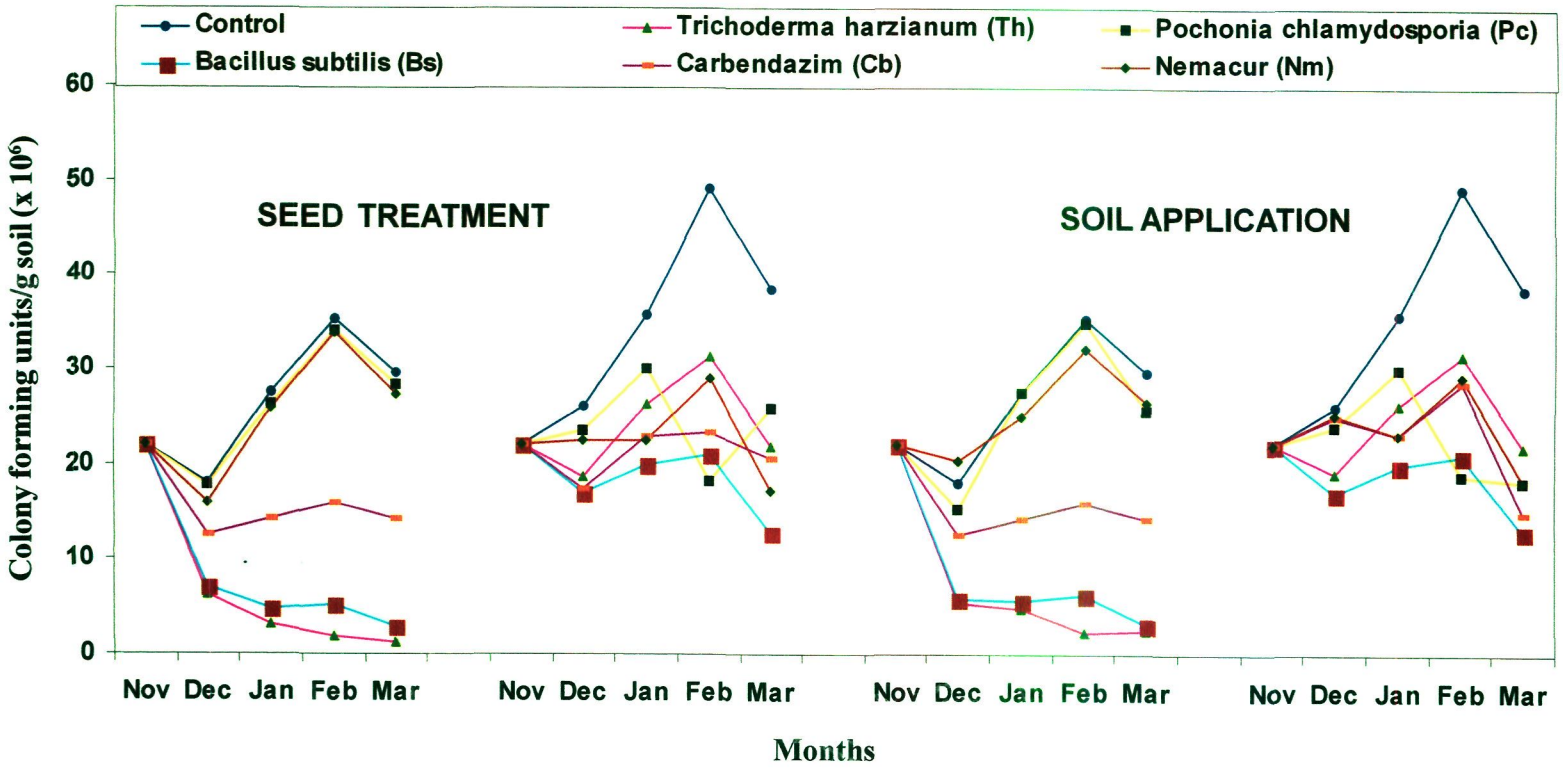
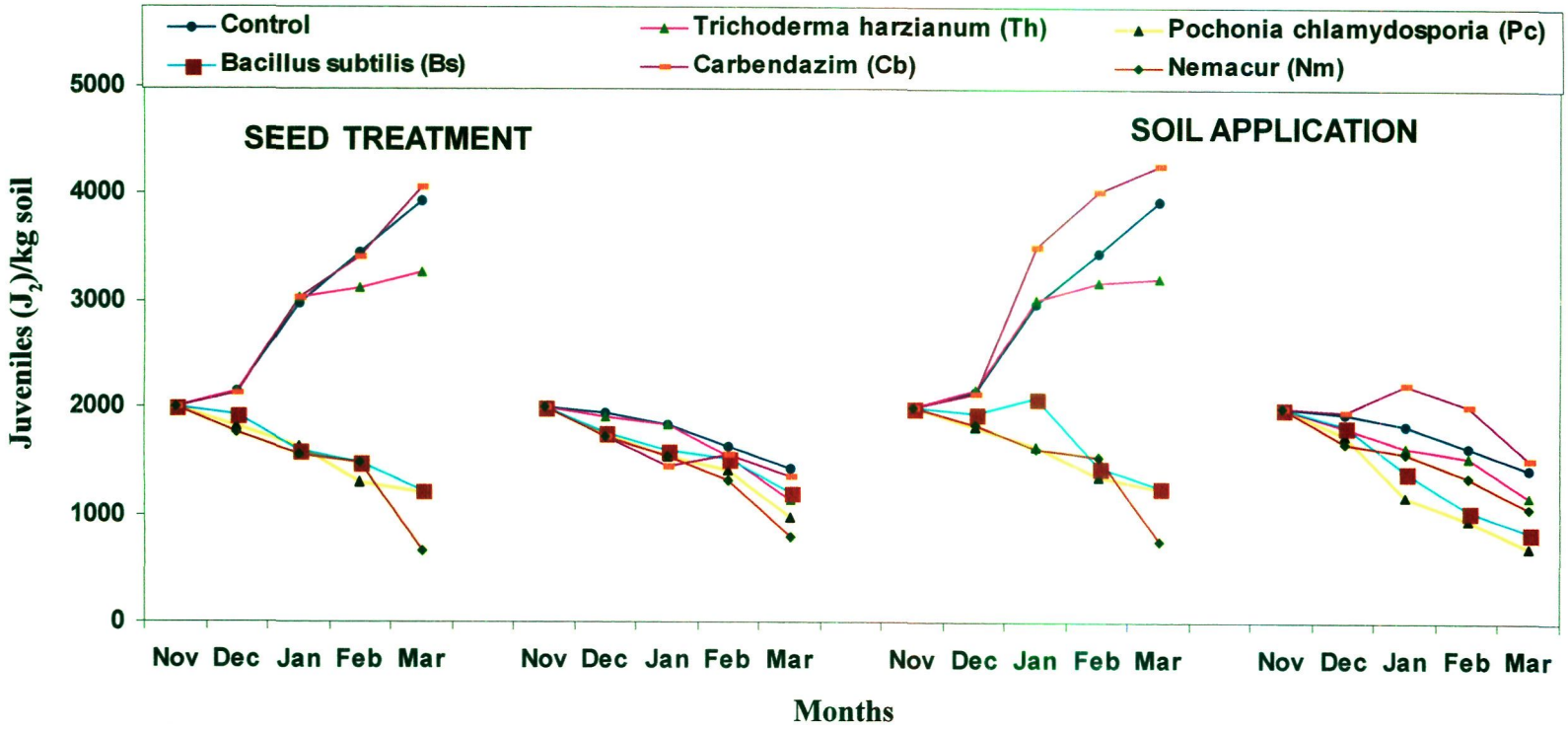


Figure 30. Effects of seed treatment and soil application with bioagents on the soil population of *Meloidogyne incognita* in the presence and absence of *Fusarium oxysporum* f. sp. *ciceri*.



nematode population 13-32% as compared to preplant population ($P \leq 0.05$). Effect of soil application of biocontrol agents on nematode population was more or less similar to seed treatment (Fig. 30).

Rhizosphere population of bioagents

Trichoderma harzianum

A 71-96% increase in the rhizosphere population of *T. harzianum* in comparison to pre-plant population (November) was recorded (Fig. 31). In the plots, where *F. oxysporum* f. sp. *ciceri* was inoculated, population of *T. harzianum* was increased by 55-107% as compared to pre plant treatment but decreased in comparison to respective month controls. Similar trend was found in the plots where root-knot nematode and wilt fungus were inoculated concomitantly and population increase was 84-102% in comparison to pre plant population but was decreased with respective month control. Effect of soil application was more or less identical (Fig. 31). Increase in the population in nematode infested plots was 84-102% in comparison to the control.

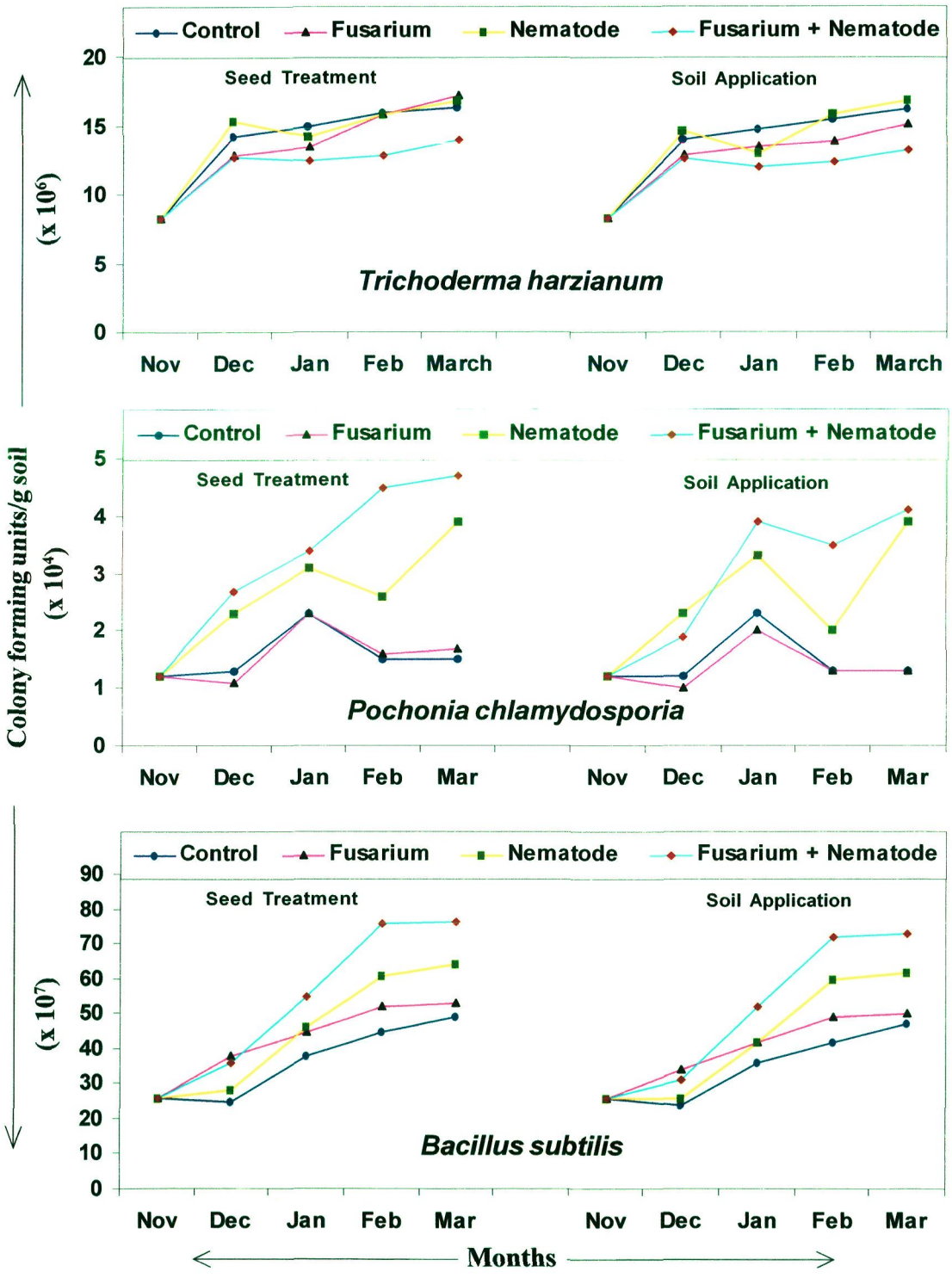
Pochonia chlamydosporia

Rhizosphere population of *P. chlamydosporia* was increased by 8.3 - 25% with a greatest increase of upto 92% during January (Fig. 31). In the presence of pathogenic fungus, *P. chlamydosporia* population was increased by 6-92% and 33-42% in comparison to pre plant and respective month controls, respectively. Rhizosphere population of the fungus was 92-225 and 92-200% greater in the plots infested with *M. incognita* alone in comparison to the preplant population and respective month control, respectively. In concomitantly inoculated treatments, rhizosphere population of *P. chlamydosporia* was drastically increased (48-213%). Effect of soil application was also same (Fig. 31).

Bacillus subtilis

An increase of 46-89% in the rhizosphere population of *B. subtilis* was recorded during the course of experiment except in December (Fig. 31). In the plots where *F. oxysporum* f. sp. *ciceri* was inoculated, population of *B. subtilis* increased significantly and the percent increase varied 8-52% in various treatments. In nematode alone and concomitantly inoculated treatments, population of *B. subtilis* also increased significantly but greater than other treatments. Effect of soil application was more or less identical to seed treatment (Fig. 31).

Figure 31. Rhizosphere population of biocontrol agents in relation to single or concomitant inoculations with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.



Experiment IV

DEVELOPMENT OF BIOPESTICIDES OF *TRICHODERMA HARZIANUM*, *POCHONIA CHLAMYDOSPORIA* AND *BACILLUS SUBTILIS*

In view of effectiveness of *Trichoderma harzianum*, *Pochonia chlamydosporia* and *Bacillus subtilis* against wilt, root-knot and disease complex of chickpea tested under field condition, their biopesticides were developed.

Production of mass/stock culture of biocontrol agents

Among various agricultural and waste materials viz., seed husk-soil-molasses, saw dust-soil-molasses, bagasse-soil-molasses, leaf litter-molasses, sorghum meal-molasses and sorghum seeds tested for mass production of biocontrol fungi and bacteria greatest CFU load of the microorganisms was recorded on sawdust-soil-molasses 5% mixture (SSM) in the ratio of 15:5:1. Hence this material was selected to grow mass culture (stock culture) of *Trichoderma harzianum*, *Pochonia chlamydosporia* and *Bacillus subtilis*.

Immobilization of biocontrol agents

Various immobilizing agents viz., talc, charcoal, fine clay and fly ash were selected for further study (Fig. 32). The stock culture of biocontrol fungi viz., *T. harzianum, P. chlamydosporia* and *B. subtilis* was mixed in the above mentioned four carriers and 5% molasses in the ratio of 1:0:0, 1:5:1, 1:10:1 and 1:20:1 (stock culture : carrier : molasses). After 15 days of incubation, the fly ash based formulation revealed highest CFU count in comparison to the other materials used (Fig. 32). The CFU load of *T. harzianum, P. chlamydosporia* and *B. subtilis* increased by 31-117%, 19-40% and 35-78% in the fly ash based carrier compared to the stock culture or other carriers (Fig. 32).

Final composition of the biopesticides

A mixture of fly ash, soil (loam) and 5% molasses in the ratio of 15:3:1 plus 10 mg chloramphenicol/kg formulation for biocontrol fungi was used as a carrier to immobilize *T. harzianum, P. chlamydosporia*. Thereafter, stock culture was mixed with this carrier in the ratio of 1:5; 1:10; 1:15 and 1:20 and filled in polybags. After

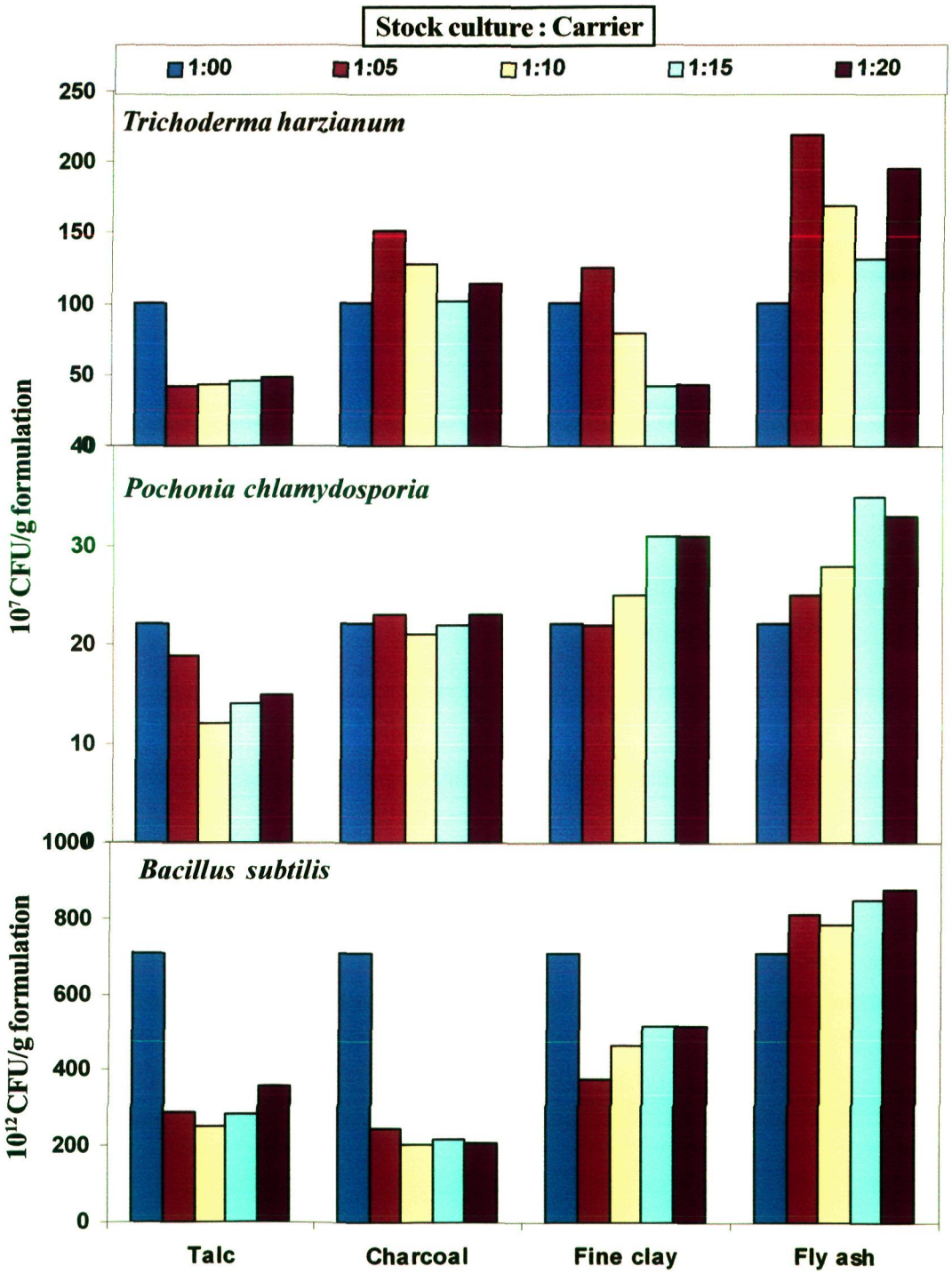


Figure 32. Multiplication of biocontrol agents in talc, charcoal, fine clay and flyash.

15 days of incubation greatest CFUs/g formulation was recorded in the formulation containing 20 parts fly ash and 1 part stock culture in comparison to 1:5, 1:10 and 1:15. The formulations were packed in airtight polypags of 200, 500 and 1000 g. The formulations were named as Biowilt-X (*Trichoderma harzianum*), Bionem-X (*Pochonia chlamydosporia*) and Biocure-X (*Bacillus subtilis*).

Shelf life

The shelf life test of the three biopesticides at five temperature regimes i.e., 5°C, 10°C, 15°C, 25°C and ambient (Febuary to September) for 32 weeks revealed that the biocontrol fungi and bacteria remained viable and also multiplied during storage, evidenced by a much greater CFU load during the storage. The CFU load of the biocontrol agents during storage is summarized under the following headings supported by (Fig. 33).

Biowilt-X (*Trichoderma harzianum*): At ambient temperature, the CFU count of *T. harzianum* increased significantly in comparison to other temperatures, next temperature in yielding higher CFUs was 25°C. Greatest CFU load/g formulation (10^{10}) was recorded during 4 to 12 weeks (Fig. 33). From 12th week onwards, the CFU count gradually declined but even at 16th week of storage at 25°C or ambient temperature it was greater than the control (pre storage value). The fungus was detected in the formulation upto 32 weeks.

Bionem-X (*Pochonia chlamydosporia*): The CFU count of *P. chlamydosporia* in the formulation was greater during 4-14 weeks and attained a peak of 3-4 x 10^9 CFUs at 10th or 12th week at 25°C or ambient temperature (Fig. 33). Thereafter, the CFU count drastically decreased but still it was at par with the control (pre storage value) at 32nd week of storage at 25°C or ambient. At rest of the temperature regimes, the biocontrol fungus was not detected after 14 weeks.

Biocure-X (*Bacillus subtilis*): The CFU load of *B. subtilis* increased from first week reaching to its peak at 2-12 weeks (9-12 x 10^{13}) at 25°C or ambient temperature (Fig. 33). From 12th week onwards it gradually decreased to a minimum at the 32nd week, but still the CFU count was equal to the control (pre storage value). At rest of the temperatures the CFU load in the formulation was much lower than the control.

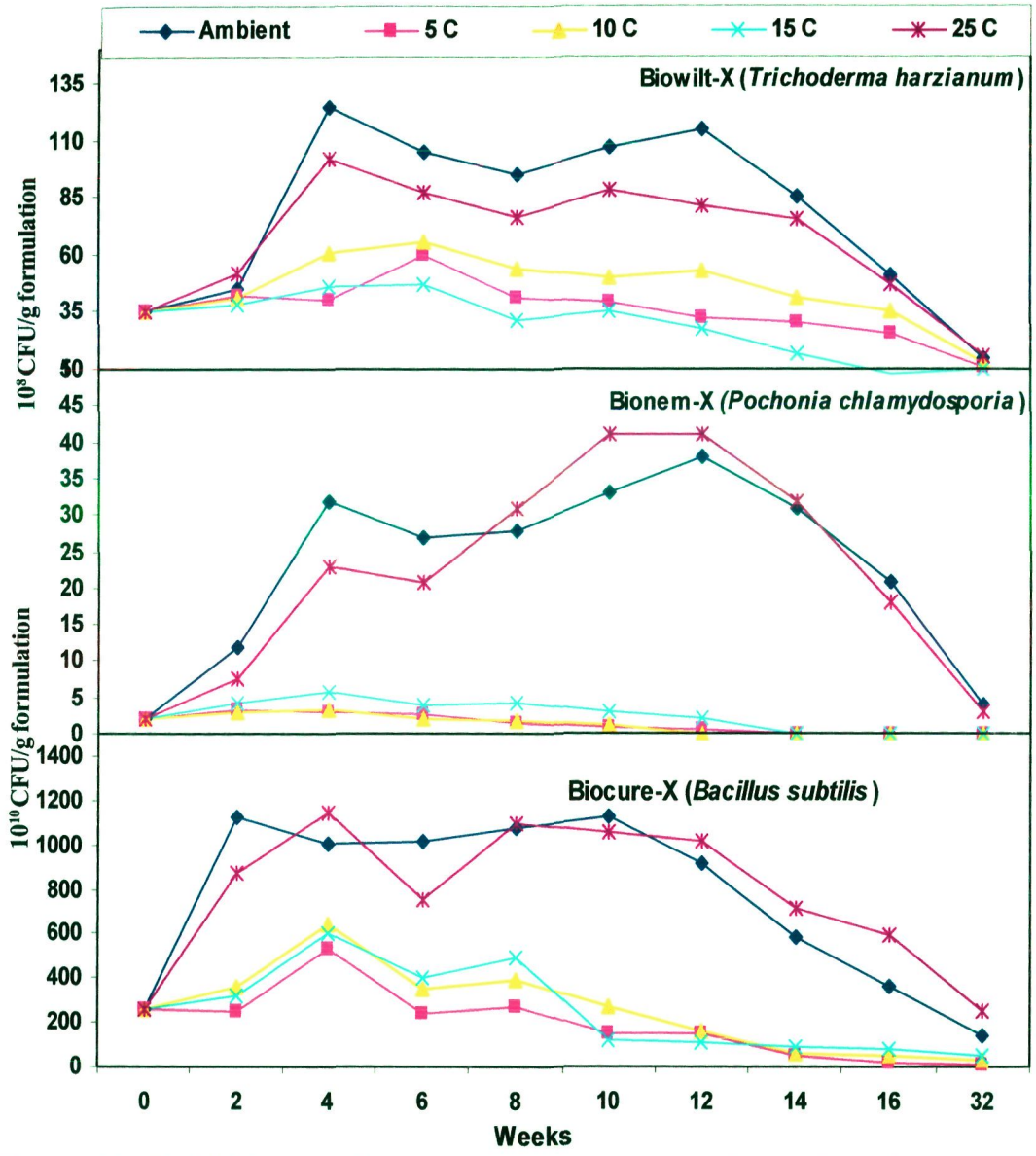


Figure 33. Shelf life test of the biocontrol agents showing colony forming units per gram formulation at various storage temperatures and durations.

Experiment V

FIELD TRIAL FOR EVALUATION OF NEWLY DEVELOPED BIOPESTICIDES FOR EFFECTIVENESS AGAINST FUSARIAL WILT, ROOT-KNOT AND DISEASE COMPLEX OF CHICKPEA

Newly developed biopesticides, viz., Biowilt-X (*Trichoderma harzianum*), Bionem-X (*Pochonia chlamydosporia*) and Biocure-X (*Bacillus subtilis*) were applied to seeds (5 g/kg seed) and soil (40 g/microplot) to evaluate their effectiveness against the target diseases under field condition.

Symptoms

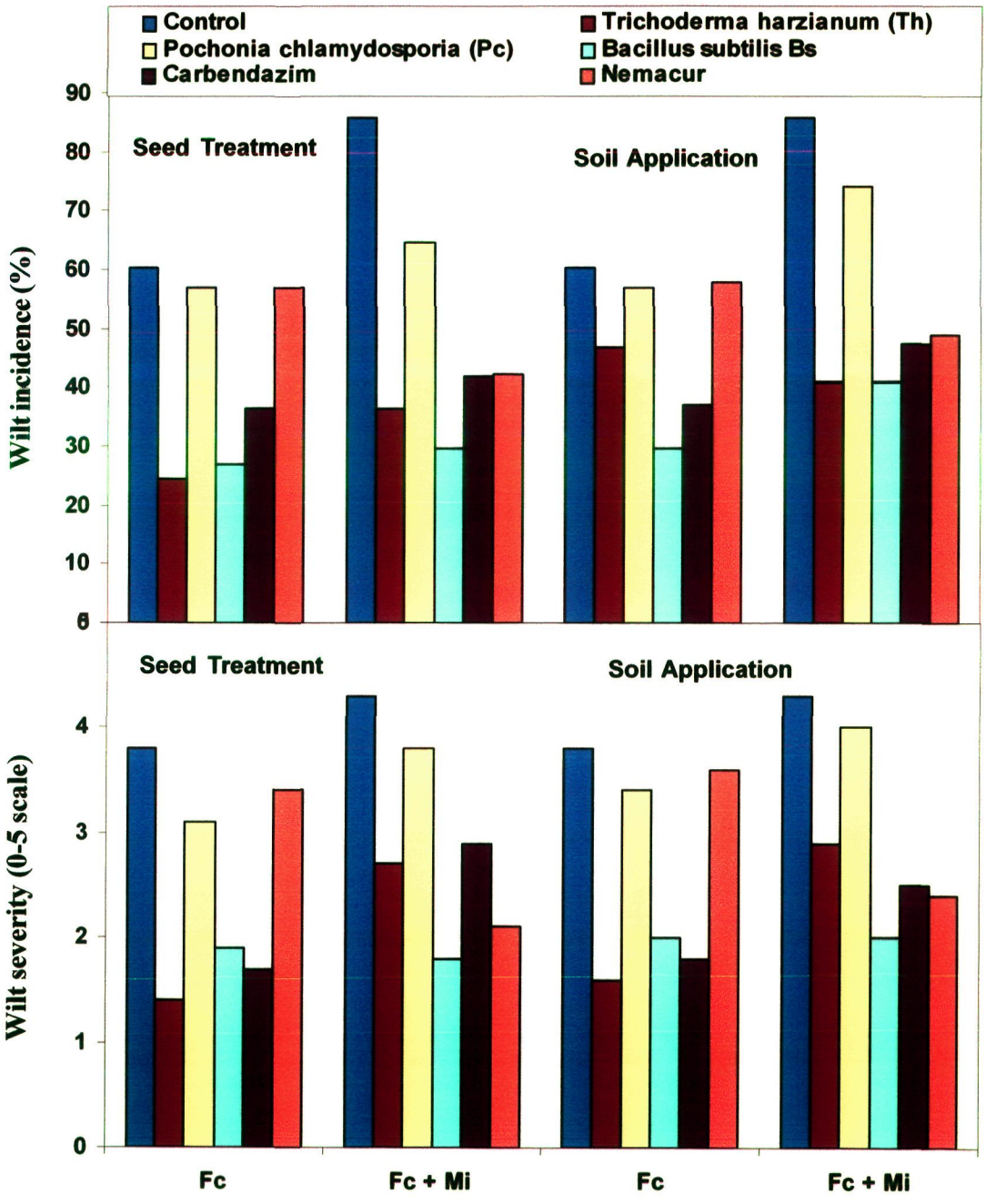
Fusarial wilt

Characteristic symptoms of wilt developed in the plots inoculated with *Fusarium oxysporum* f. sp. *ciceri*. Incidence of the wilt was 60% with 75% wilt severity (Fig. 34 & 35). The wilting was, however, checked due to application of various treatments but to a varying extent. Seed treatment and soil application of Biowilt-X (*T. harzianum*) decreased the wilt incidence by 60 and 53% and the severity by 63 and 58%, respectively over respective control (Fig. 34 & 36). Treatment with the biopesticide, Biocure-X (*B. subtilis*) was next in effectiveness and decreased the wilting by 51-55% (seed treatment) and 47-51% (soil application) in comparison to respective controls (Fig. 34 & 36). Effect of carbendazim was significant ($P \leq 0.05$) in checking the wilt symptoms but much lesser effective than the biopesticides.

Root-knot

Root-knot nematode, *Meloidogyne incognita* caused 78 galls and 67 egg masses/root system of chickpea plants (Fig. 37). Both Bionem-X (*P. chlamydosporia*) and Biocure-X (*B. subtilis*) significantly ($P \leq 0.05$) decreased the number of galls and egg masses per root system with marginal difference in seed (20-25%) and soil treatments (20-24%). Application of nemacur supressed galling and egg mass production by 12 and 18% (soil application), and 11 and 18% (seed treatment) (Fig. 37).

Figure 34. Effects of seed treatment and soil application of of newly developed biopesticides on the incidence and severity of wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceri* in the presence and absence of *Meloidogyne incognita.*



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

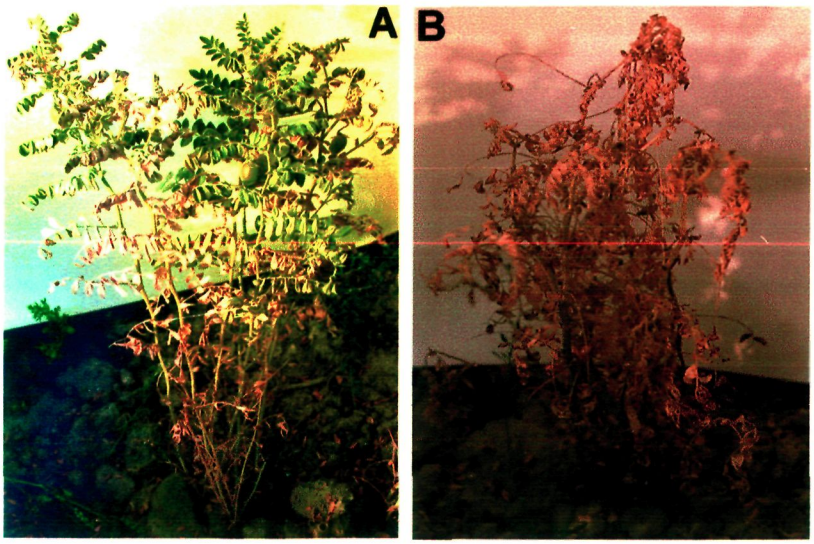


Figure 35. Chickpea plants showing wilt symptoms caused by *Fusarium oxysporum* f. sp. *ciceri* Moderate (A) and severe (B) wilting, browning and drying of foliage.

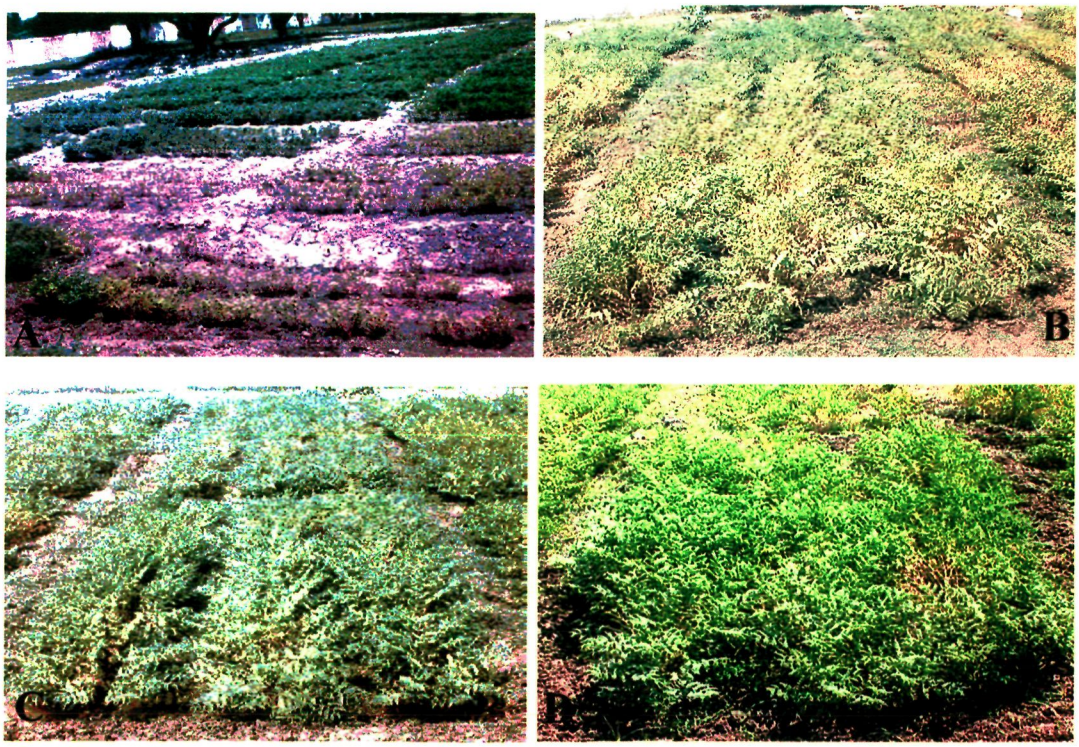
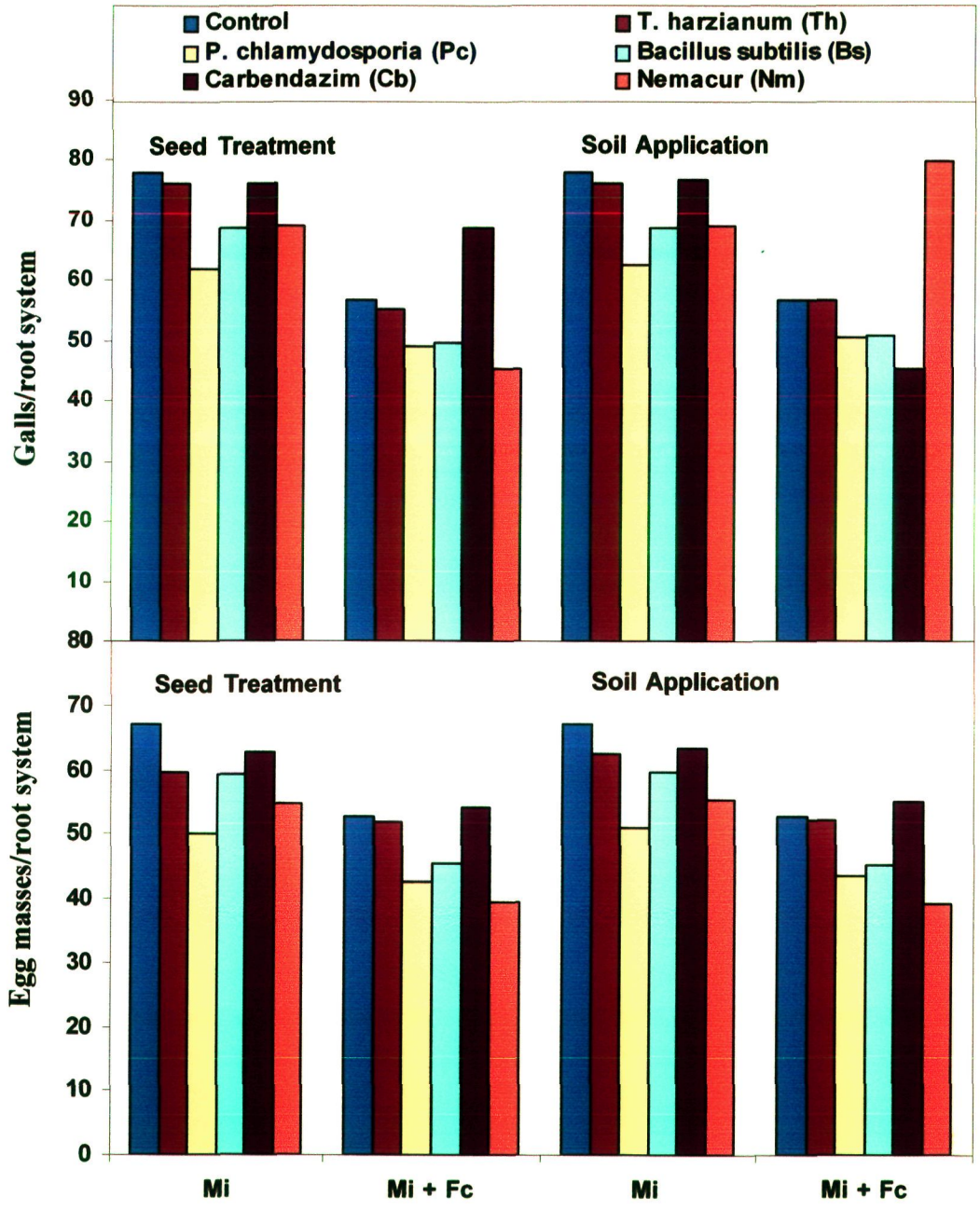


Figure 36. Effect of seed treatment with biopesticides on fusarial wilt. - (A) Microplots showing infection of wilt in the presence or absence of root-knot nematode. Management of the disease by application of Biowilt -X (*T. harzianum*) (B) and Biocure-X (*B. subtilis*) (C & D).

Figure 37. Effects of seed treatment and soil application of newly developed biopesticides on the galling caused by *Meloidogyne incognita* in chickpea in the presence and absence of *Fusarium oxysporum* f. sp. *ciceri.*



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

Disease complex

Concomitant inoculation with *F. oxysporum* f. sp. *ciceri* and *M. incognita* caused greater symptoms of fusarial wilt compared to *F. oxysporum* f. sp. *ciceri* alone (Fig. 34). Seed treatment with *B. subtilis* or *T. harzianum* biopesticide decreased the wilt incidence by 65 and 57%, and severity by 59 and 37%, respectively over control. Soil application of the biopesticides was 2-6% less effective than the seed treatment (Fig. 34).

Chickpea plants grown in concomitantly inoculated plots developed significantly lesser number of galls and egg masses than those grown in the plots infested with the nematode alone. Application of Bionem-X (*P. chlamydosporia*) or Biocure-X (*B. subtilis*) suppressed the gall formation and egg mass production by 13-19% and 12-14%, respectively over control (Fig. 37). Among the pesticides, greatest decrease in gall and egg mass count occurred with nemacur being 20-26% with soil and seed treatments, respectively (Fig. 37).

Dry matter production and yield

Dry matter production and yield of chickpea increased significantly ($P \leq 0.05$) with the application of Biocure-X (*B. subtilis*) and Biowilt-X (*T. harzianum*) on seeds or in soil. Seed treatment showed greater yield enhancement than soil application. Other treatments also provide significant yield enhancement in control plots (without pathogens). In the plots where *F. oxysporum* f. sp. *ciceri* was inoculated, 18-19% suppression in the dry matter production and yield of chickpea was observed (Table 27). Application of biopesticides or pesticides checked the suppressive effect of the pathogens resulting to promotion in the plant growth and yield. Seed treatment with Biowilt-X or Biocure-X enhanced the dry matter production and yield by 46-55% (Table 27). Carbendazim application promoted the yield by 21% (seed treatment) and 23% (soil application) over control.

Plants growing in nematode inoculated plots produced dry matter production and yield less 13 and 15% than control (Table 27). Seed treatment with all the three biopesticides suppressed the pathogenic effect of the nematode and increased both the variables over control ($P \leq 0.05$). Greatest yield enhancement occurred with Biocure-X followed by Bionem-X and Biowilt-X which were significantly different at $P \leq 0.05$. Soil application of nemacur increased the yield and dry matter production by 17-20% in comparison to the respective control (Table 27).

Table 27. Effects of seed treatment and soil application of newly developed biopesticides on the dry matter production and yield of chickpea in plots infested singly or concomitantly with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.

Treatment	Shoot dry weight (g)		Yield per plant (g)	
	Seed Treatment	Soil Application	Seed Treatment	Soil Application
(C1)	23.9	23.9	7.5	7.5
T. harzianum (Th)	30.9 (29.3)	29.5 (23.4)	8.6 (14.7)	8.4 (12.0)
P. chlamydosporia (Pc)	26.2 (9.2)	25.4 (6.3)	8.1 (8.0)	8.2 (9.3)
B. subtilis (Bs)	33.5 (40.2)	32.0 (33.9)	9.8 (30.7)	9.9 (32.0)
Carbendazim (Cb)	26.7 (11.7)	26.5 (10.9)	8.1 (8.0)	8.0 (6.7)
Nemacur (Nm)	26.9 (12.6)	27.4 (14.6)	8.0 (6.7)	8.1 (8.0)
(C2)	19.5 (-18.4)	19.5 (-18.4)	6.1 (-18.7)	6.1 (-18.7)
T. harzianum + F	28.7 (47.2)	27.8 (42.6)	8.9 (45.9)	8.5 (39.3)
P. chlamydosporia + F	21.4 (9.7)	22.0 (12.8)	6.5 (6.6)	6.2 (1.6)
B. subtilis + F	30.3 (55.4)	30.0 (53.8)	9.4 (54.1)	9.2 (50.8)
Carbendazim + F	24.3 (24.6)	24.0 (23.1)	7.4 (21.3)	7.5 (22.9)
Nemacur + F	20.1 (3.1)	19.8 (1.5)	6.7 (9.8)	6.5 (6.6)
(C3)	20.9 (-12.6)	20.9 (-12.6)	6.4 (-14.7)	6.4 (-14.7)
T. harzianum + N	23.1 (10.5)	23.2 (11.0)	7.1 (10.9)	7.2 (12.5)
P. chlamydosporia + N	26.7 (27.8)	25.5 (22.0)	8.2 (28.1)	8.0 (25.0)
B. subtilis + N	27.8 (33.0)	27.5 (31.6)	8.6 (34.4)	8.2 (28.1)
Carbendazim + N	22.4 (7.2)	21.7 (3.8)	7.0 (9.3)	6.6 (3.1)
Nemacur + N	24.5 (17.2)	25.0 (19.6)	7.6 (18.7)	7.4 (15.6)
(C4)	14.6 (-38.9)	14.6 (-38.9)	4.1 (-45.3)	4.1 (-45.3)
Th+ FN	18.4 (26.0)	18.0 (23.3)	4.8 (17.1)	5.0 (21.9)

Continued.....

Continued..... Table 27

Pc + FN	17.3 (18.5)	17.1 (17.1)	4.7 (14.6)	4.2 (2.4)
Bs + FN	22.6 (54.8)	22.0 (50.7)	6.1 (48.8)	5.9 (43.9)
Cb + FN	19.1 (30.8)	18.1 (24.0)	4.6 (12.2)	4.5 (9.8)
Nm + FN	19.4 (32.9)	19.1 (30.8)	4.9 (19.5)	5.1 (24.4)
LSD ($P \leq 0.05$)	0.27	0.28	0.05	0.10
F-value $P \leq 0.05$				
Control agents (df = 5)	446.87*	359.20*	1048.47*	224.49*
Pathogens (df = 1)	59.97*	95.95*	1827.07*	309.31*
Interaction (df = 5)	14.01*	17.10*	33.49*	NS

Values in parenthesis are percent increase (+ve) or decrease (-ve) over respective control (Plants not inoculated with either pathogen but applied with biocontrol agents were compared with Uninoculated Control C1; wilt fungus inoculated plants applied with biocontrol agents were compared with fungus inoculated control C2; nematode inoculated plants applied with biocontrol agents were compared with nematode inoculated control C3; concomitantly inoculated plants applied with biocontrol agents were compared with concomitantly inoculated control C4). * Significantly different from the control at $P \leq 0.05$; NS- Not significant at $P \leq 0.05$.

Concomitant inoculation with *F. oxysporum* f. sp. *ciceri* and *M. incognita* caused a decrease of 39-45% in the dry matter production (Table 27). Greatest increase in the yield of concomitantly inoculated plants was obtained due to seed application with Biocure-X (49%). Seed treatment with carbendazim and nemacur improved the yield by 12-19% in comparison to the concomitantly inoculated control (Table 27). Soil application of biopesticides was found more or less equally effectiveness in controlling the disease.

Yield and cost-benefit ratio

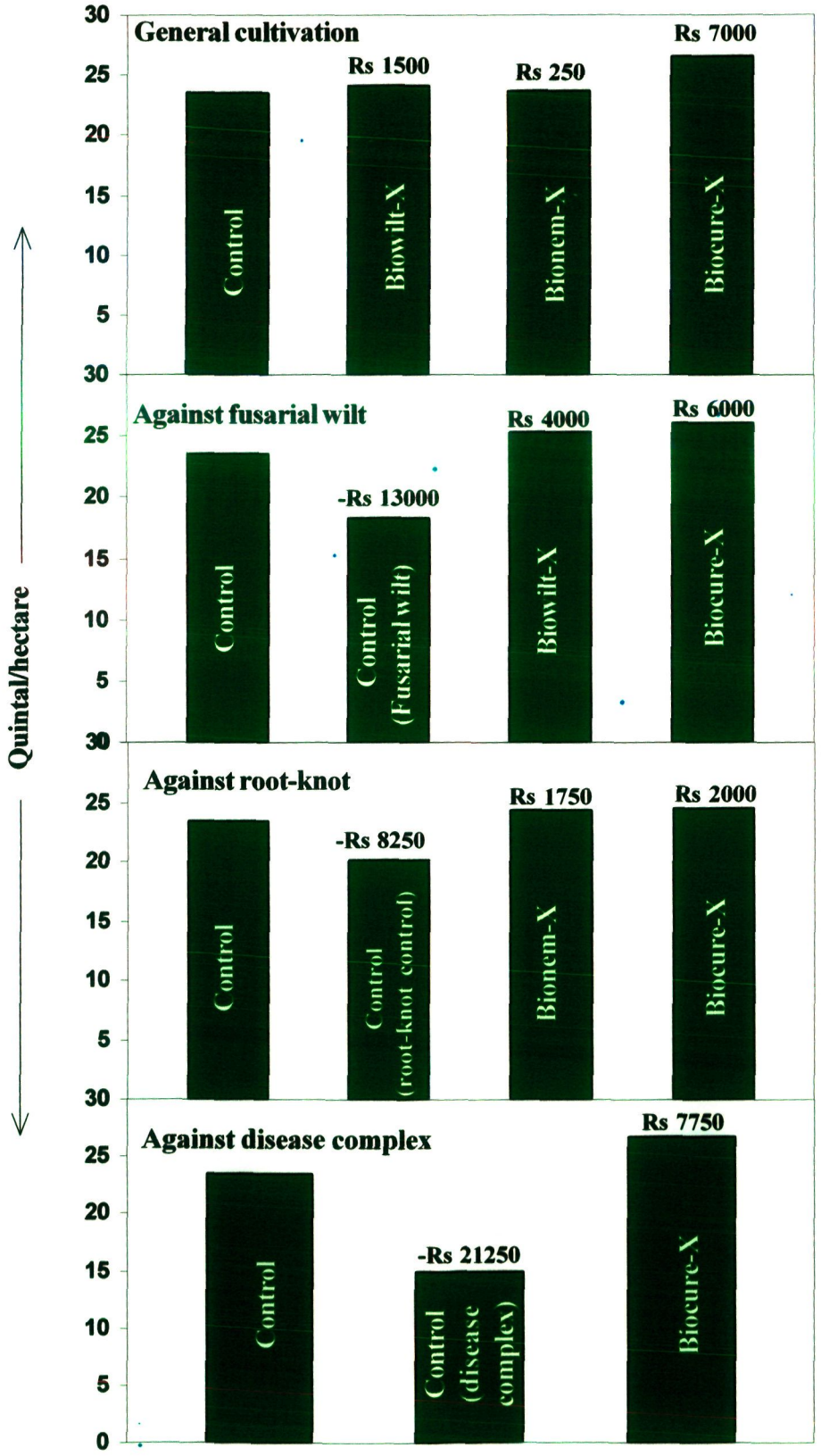
General cultivation: In the control plots, chickpea cv. BG-256 gave an yield of 23.5 q/ha which is within the prescribed yield of the cultivar used. Application of biopesticides improved productivity of the cultivar and a significant increase in the yield of chickpea was recorded with Biocure-X (*B. subtilis*). The seed treatment 5g Biocure-X/kg seed resulted to 3 q additional yield per hectare worth Rs. 7000 (approx. US $ 160.0)/ha after excluding Rs 500/ha as the cost of biopesticide application (Fig. 38).

Fusarial wilt: The wilt disease caused by *F. oxysporum* f. sp. *ciceri* inflicted 5.2 q yield loss/ha valuing Rs 13,000 (approx. US $ 300.0)/ha. The Seed treatment with Biowilt-X (*T. harzianum*) or Biocure-X (*B. subtilis*) improved the yield of infected chickpea crop resulting to a profit of Rs. 4000 (approx. US $ 100.0) and Rs. 6000 (approx. US $ 150.0)/ha in comparison to the control (Fig. 38).

Root-knot nematode: Root-knot disease caused by *M. incognita* resulted to a decrease in the yield of chickpea by 3.3 q/ha costing a monetary loss of Rs 8250(approx. US $ 200.0)/ha (Fig. 38). Seed treatment with Bionem-X (*Pochonia chlamydosporia*) or Biocure-X (*B. subtilis*) biopesticides improved the yield equivalent to Rs. 1750-2000 (approx. US $ 40-50)/ha.

Disease complex: The disease complex caused by *F. oxysporum* f. sp. *ciceri* and *M. incognita* concomitantly greatly reduced the yield of chickpea (8.5 q/ha) valuing Rs 21250 (approx. US $ 5000.0)/ha (Fig. 38). The Biocure-X (*B. subtilis*) was found effective in decreasing the suppressive effect of the pathogens and enhancing

Figure 38. Yield and cost benefit ratio of seed treatment with biopesticides in chickpea (chickpea value @ 2500/q; Indian Rs. 100= US $ 2.27).



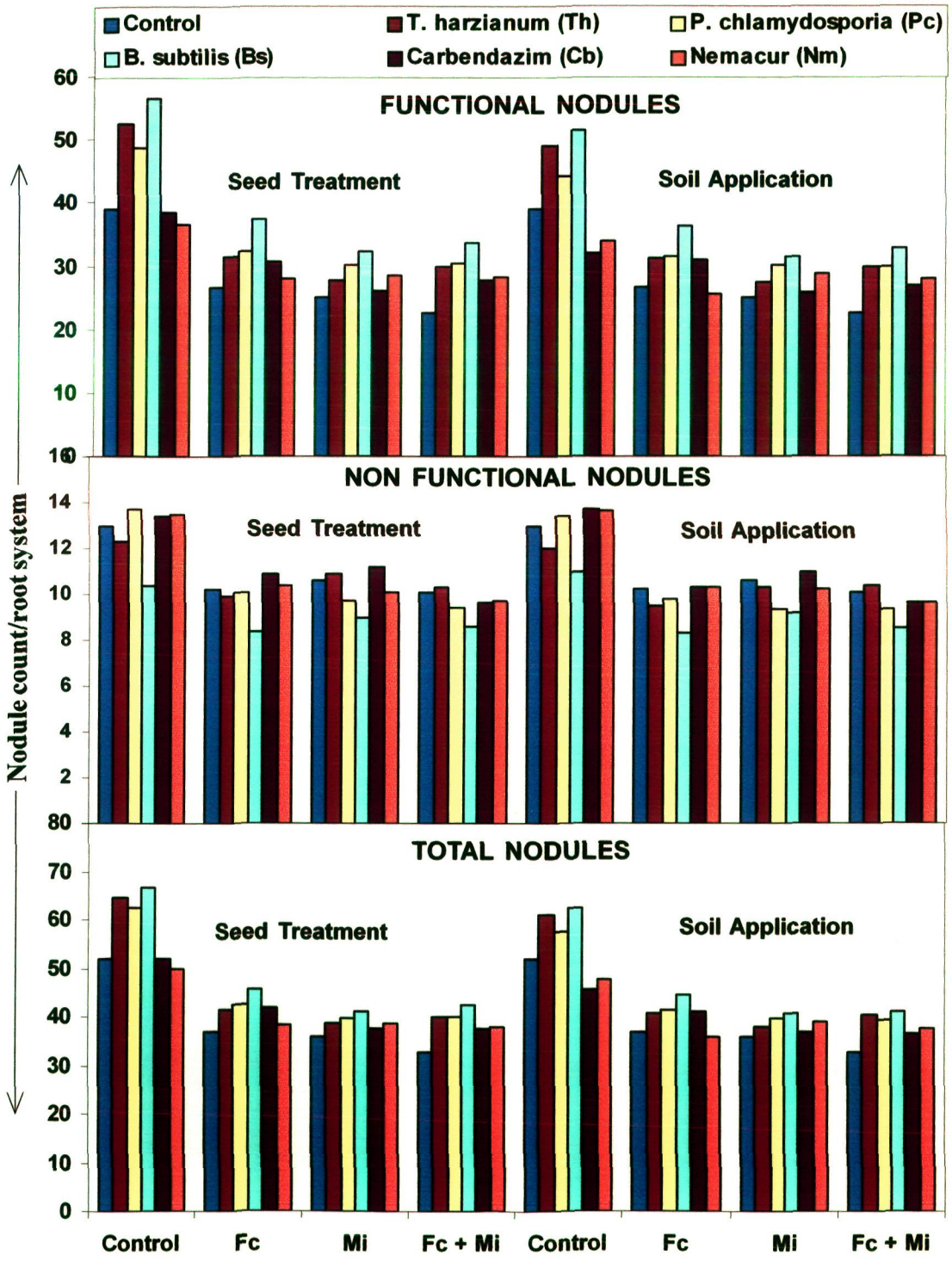
the yield. Seed treatment with Biocure-X gave a profit of Rs. 7750 (approx. US $ 175.0)/ha in chickpea plots infested with the wilt fungus and nematode concomitantly in comparison to the untreated plots (Fig. 38).

Root nodulation

On an average 52 nodules/root system were formed. Out of them 39 were functional and the remaining non functional (Fig. 39). Biocure-X was found more effective in increasing the nodulation as compared to other treatments. Seed treatment and soil application of Biocure-X caused 28 and 20% increase in total nodules of chickpea as compared to uninoculated control. Biowilt-X was next in effectiveness and increased the total nodules by 24 and 17% as compared to uninoculated control. Infection by the wilt fungus suppressed the functional and total nodules by 32 and 29%, respectively. Incorporation of various treatments decreased the suppressive effect of the pathogen on root nodulation. Application of Biocure-X on seed or in soil resulted to 40 and 36% increase in the functional nodules of fungus infected chickpea over the infected control. Increase in the functional nodules due to seed treatment with Biowilt-X was 19%. Carbendazim promoted functional nodules by 15-16%. Suppressive effect of root-knot nematode on nodulation was relatively greater than the wilt fungus resulting to 36 and 31% decrease in the number of functional and total nodules/root system (Fig. 39). Application of Bionem-X or Biocure-X enhanced the nodulation in chickpea with marginally greater effect of the later. Effect of soil application with nemacur increased the functional and total nodule count by 15 and 9%.

Concomitant inoculation by the wilt fungus and root-knot nematode decreased functional (42%), total nodules (37%) and nonfunctional (22%) as compared to the control (Fig. 39). All treatments significantly ($P \leq 0.05$) suppressed the pathogenic effect of wilt fungus and root-knot nematode, resulting to significant increase in the nodulation. Application of Biocure-X on seed or in soil increased the functional nodules by 50 and 45%, and total nodules by 30 and 26%, respectively. Seed treatment with nemacur promote functional nodules of fungus infected plants by 25%. Effect of seed treatment with the biopesticides was relatively more effective than the soil application.

Figure 39. Effects of seed treatment and soil application of newly developed biopesticides on the root nodulation in chickpea grown in the plots infested singly or concomitantly with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.



Fc- *Fusarium oxysporum* f. sp. *ciceri*; Mi- *Meloidogyne incognita*

Strain typing analysis for *Trichoderma harzianum*

Among the 10 primers tested in the PCR amplification, 5 primers showed clear and unambiguous amplification while the rest did not give amplification of several reactions tried or produced faint or fizzy lanes. Scorable 5 RAPD primers led to amplification of 127 fragments. DNA amplification patterns as detected by some of the RAPD primers have been provided in (Fig. 40). Highest genetic similarity (0.97 i.e., 97%) was measured between standard strain, Th-s and isolated strain, Th-1 and a lowest of 0.91 similarity was observed between standard strain, Th-s and isolated strain, Th-4. Among the isolated strains of *T. harzianum* the maximum genetic similarity i.e., 0.96 (between Th-2 and Th-4) and a minimum of 0.90 (between Th-5 and Th-3) were measured (Fig. 41).

Strain typing analysis for *Pochonia chlamydosporia*

In case of *P. chlamydosporia* 6 primers showed clear and unambiguous amplification among the 10 primers tested in the PCR amplification (Fig. 43), while the rest four did not give amplification of several reactions tried or produced faint or fizzy lanes. Scorable 6 RAPD primers led to amplification of 151 fragments. DNA amplification patterns as detected by some of the RAPD primers have been provided in Figure 2. Highest genetic similarity (0.93) was measured between standard strain, Pc-s and isolated strain, Pc-1 and a lowest of 0.87 similarity between standard strain, Pc-s and isolated strain, Pc-3. Among the isolated strains of *P. chlamydosporia* the maximum genetic similarity of 0.94 (between Pc-1 and Pc-2) and a minimum of 0.85 (between Pc-4 and Pc-5) were measured (Fig. 42).

Strain typing analysis for *Bacillus subtilis*

Among the 10 primers tested in the PCR amplification, 4 primers showed clear and unambiguous amplification while the rest did not give amplification of several reactions tried or produced faint or fizzy lanes. Scorable 4 RAPD primers led to amplification of 143 fragments (Fig. 44). Maximum genetic similarity of 0.87 was measured between standard strain, Bs-s and isolated strain, Bs-1 and minimum of 0.84 between Bs-s and Bs-4. Among the isolated strains of *B. subtilis* the maximum genetic similarity of 0.97 (between Bs-1 and Bs-2) and a minimum of 0.77 (between Bs-1 and Bs-4) were measured (Fig. 45).

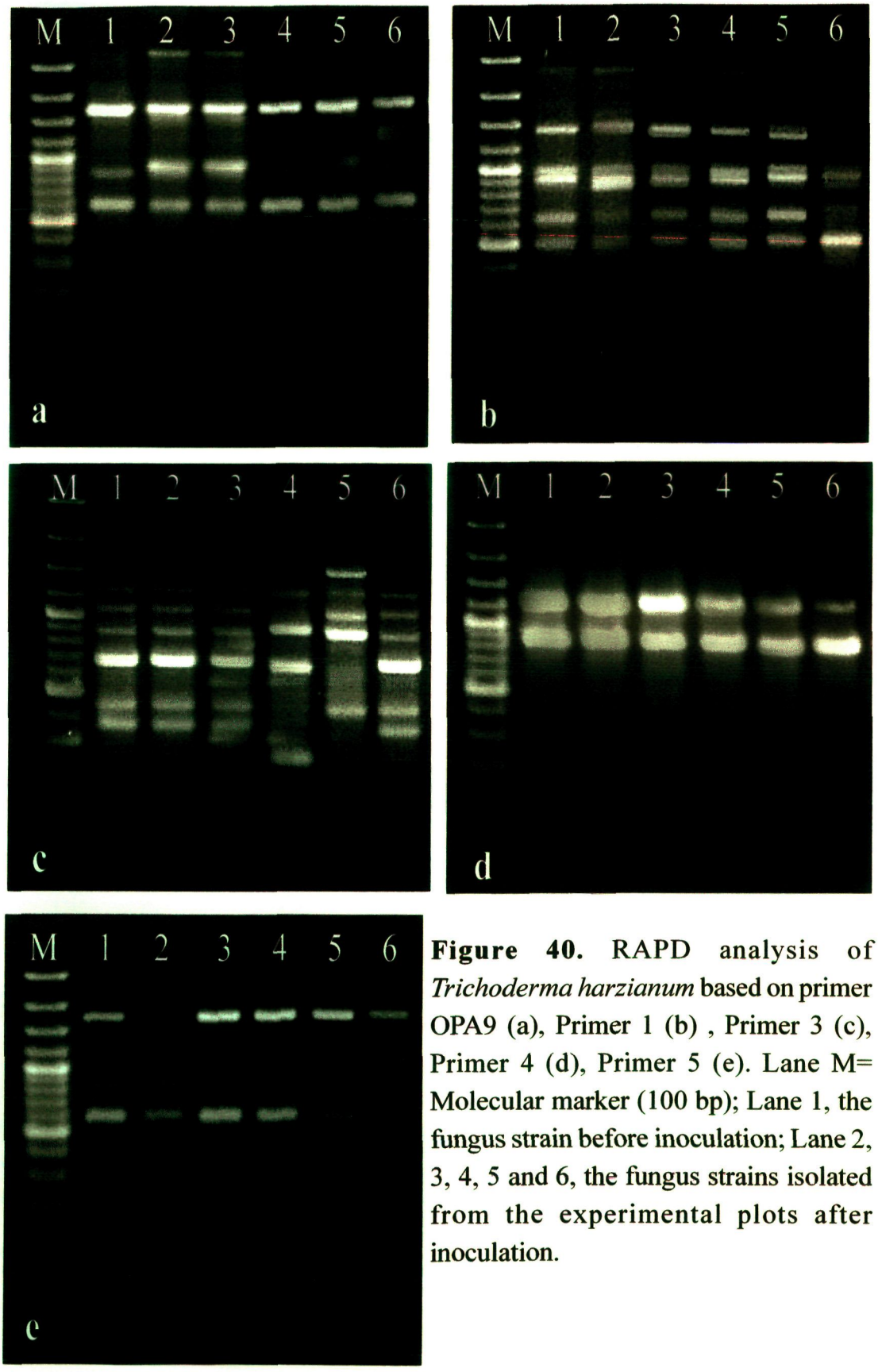


Figure 40. RAPD analysis of *Trichoderma harzianum* based on primer OPA9 (a), Primer 1 (b) , Primer 3 (c), Primer 4 (d), Primer 5 (e). Lane M= Molecular marker (100 bp); Lane 1, the fungus strain before inoculation; Lane 2, 3, 4, 5 and 6, the fungus strains isolated from the experimental plots after inoculation.

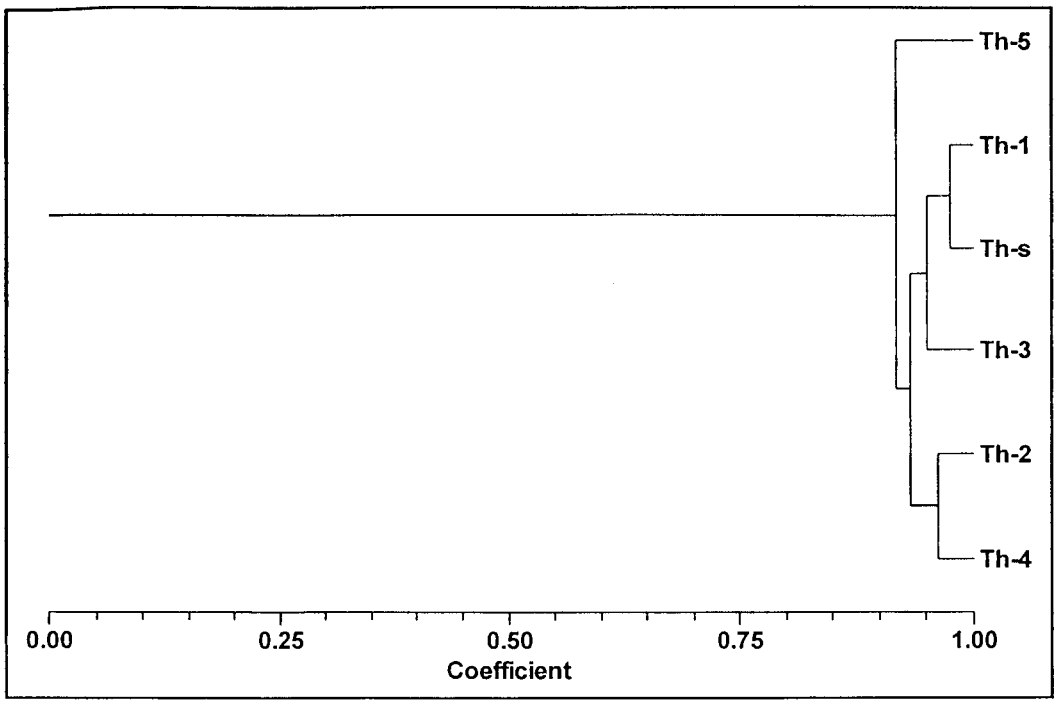


Figure 41. Dendrogram of *Trichoderma harzianum* standard strain before inoculation (Th-s) and isolated strains (Th-1, Th-2, Th-3, Th-4, Th-5), constructed using UPGMA with Jaccard's similarity index based on 10 RAPD primers.

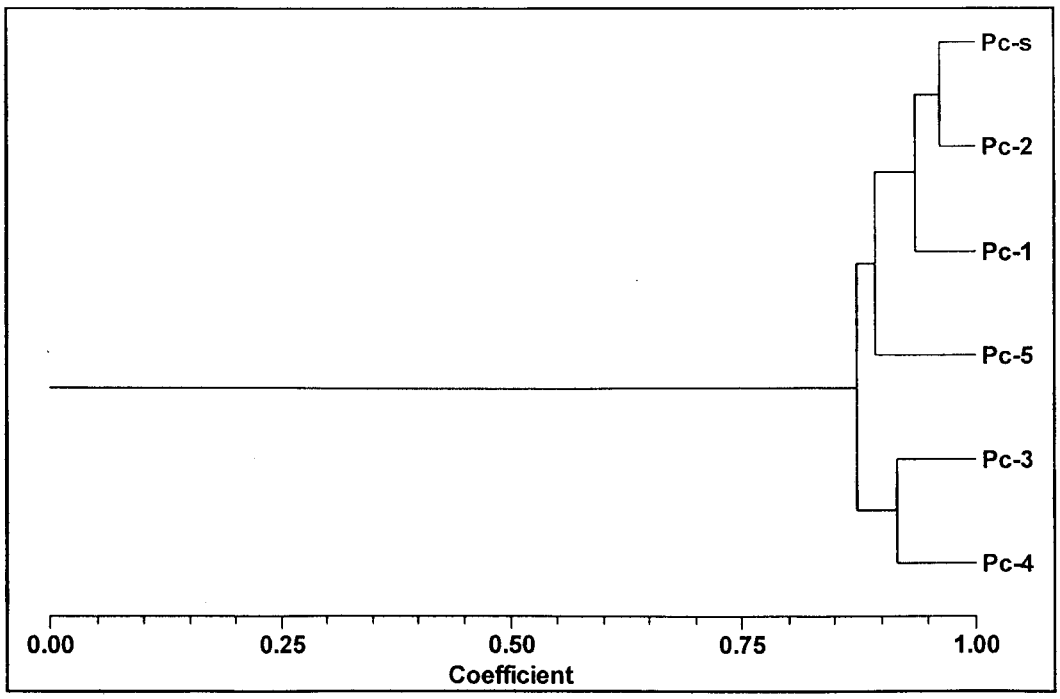


Figure 42. Dendrogram of *Pochonia chlamydosporia* standard strain before inoculation (Pc-s) and isolated strains (Pc-1, Pc-2, Pc-3, Pc-4, Pc-5), constructed using UPGMA with Jaccard's similarity index based on 10 RAPD primers.

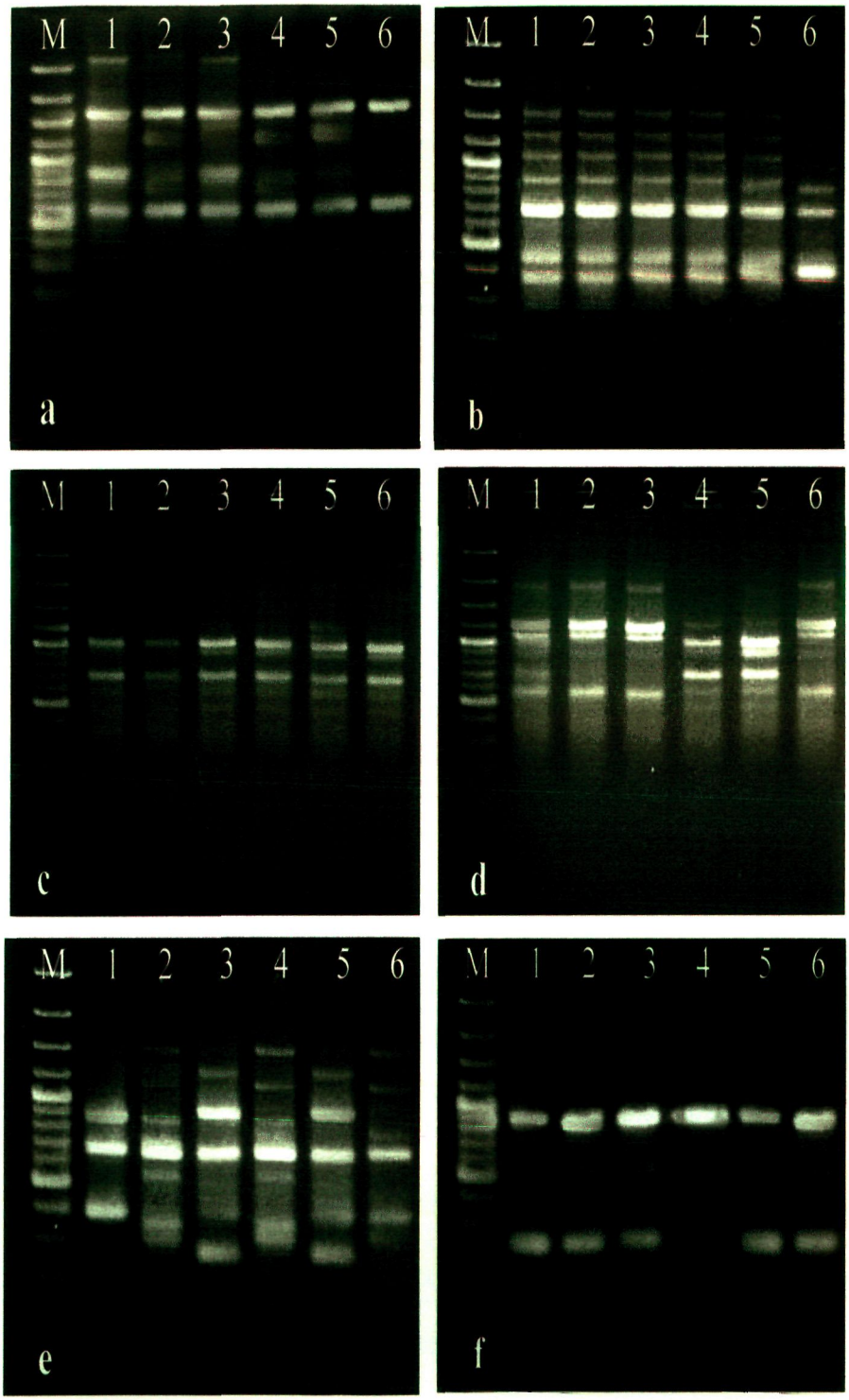


Figure 43. RAPD analysis of *Pochonia chlamydosporia* based on primer OPA 9 (a), Primer 1 (b), Primer 2 (c) , Primer 3 (d), Primer 4 (e), Primer 5 (f). Lane M= Molecular marker (100 bp); Lane 1, the fungus strain before inoculation; Lane 2, 3, 4, 5 and 6, the fungus strains isolated from the experimental plots after inoculation.

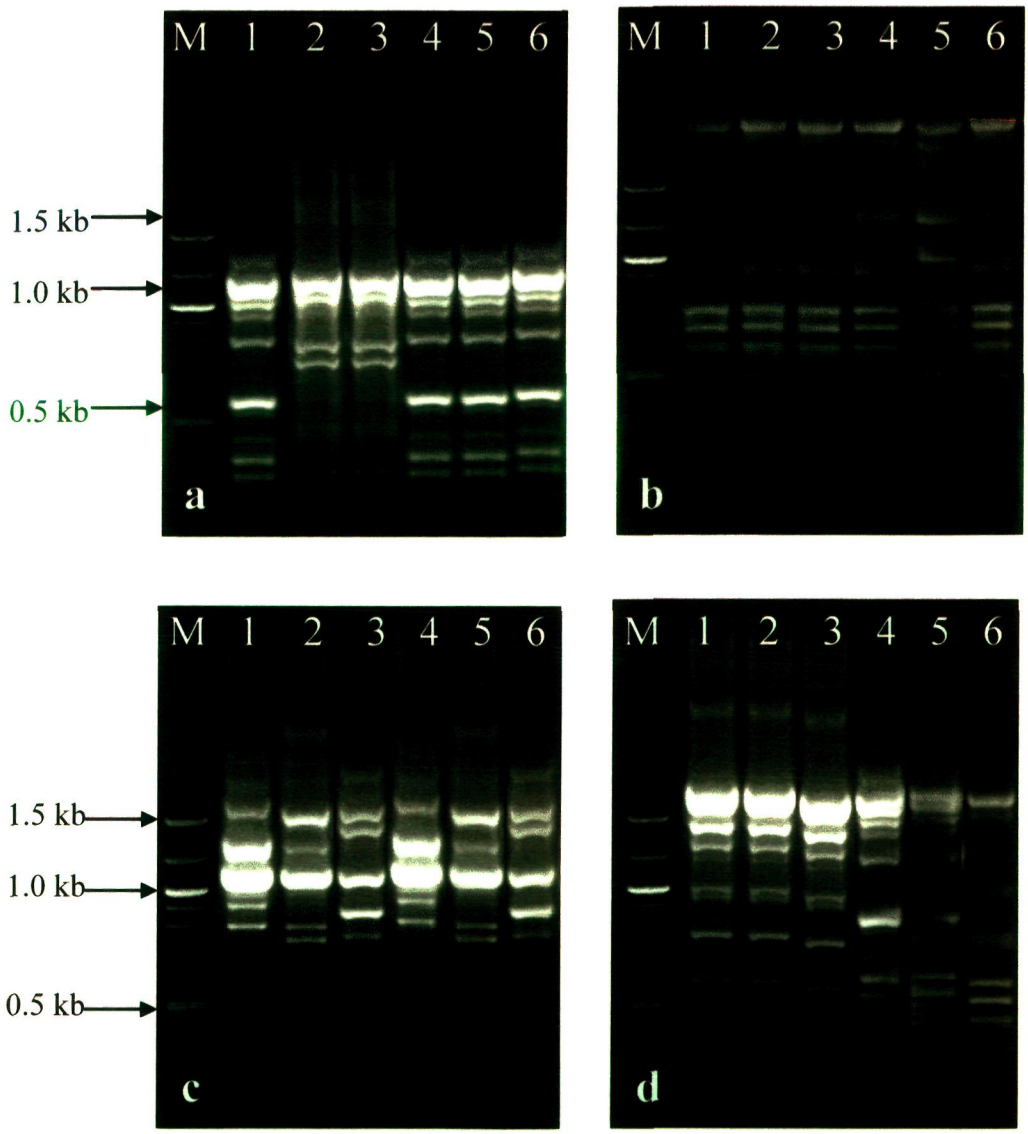


Figure 44. RAPD analysis of *Bacillus subtilis* based on Primer 6 (a), Primer 7 (b), Primer 8 (c), Primer 9 (d). Lane M= Molecular weight marker; Lane 1, the bacterial strain before inoculation; Lane 2, 3, 4, 5, 6, the bacterial strains isolated from the experimental plots after inoculation.

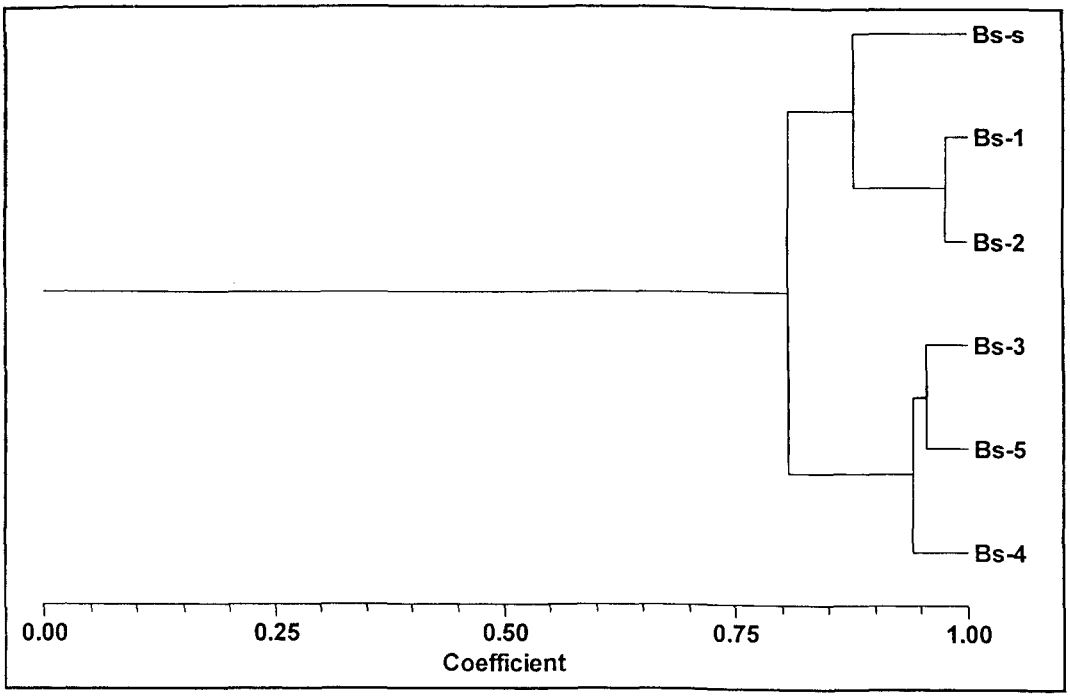


Figure 45. Dendrogram of *Bacillus subtilis* standard strain before inoculation (Bs-s) and isolated strains (Bs-1, Bs-2, Bs-3, Bs-4, Bs-5), constructed using UPGMA with Jaccard's similarity index based on 10 RAPD primers.

Rhizosphere population of the pathogens

Fusarium oxysporum f. sp. *ciceri*

In the plots where *F. oxysporum* f. sp. *ciceri* was inoculated without any other treatment, soil population of the wilt fungus increased by 22-56% in January and February and 32% in March in comparison to planting population (Fig. 46). Different treatments, however, suppressed the population of *F. oxysporum* f. sp. *ciceri* but to a varying extent. Among the biopesticide treatments applied, lowest population of wilt fungus was recorded with Biowilt-X through seed (71-93%) or soil application (70-92%) in comparison to respective month control. Treatment with Biocure-X decreased the soil population of the pathogenic fungus by 68-88%. Carbendazim application caused 35-52% decline in the population of wilt fungus in comparison to respective month control.

In the plots where root-knot nematode and wilt fungus were inoculated concomitantly, increase in the population of wilt fungus was significantly greater than in the plots where nematodes were not inoculated (Fig. 46). Seed treatment with Biocure-X, however, decreased the population of wilt fungus by 40-68% in comparison to the population of concomitantly inoculated control. Soil application was less effective than the seed treatment of biopesticides. Effect of fungicide treatment was relatively less effective than Biocure-X. Application in soil or on seed with the carbendazim and nemacur resulted to 7-47% and 11-54% decrease in the population of wilt fungus over respective month control (Fig. 46).

Meloidogyne incognita

A gradual decrease in the soil population of juveniles of *M. incognita* was recorded over the course of experiment (Fig. 47). The nematode population, however, decreased significantly in the plots where Bionem-X was applied on seeds (20-73%) or in soil (19-72%), in comparison to respective month controls. Application of Biocure-X also significantly ($P \leq 0.05$) suppressed the nematode population. Soil application or seed treatment with nemacur decreased the nematode population greater than Bionem-X.

In the presence of wilt fungus, nematode population decreased by 13-67% in comparison to the respective month control (Fig. 47). Seed treatment with Bionem-X caused greatest decline in the nematode population of concomitantly inoculated

Figure 46. Effects of seed treatment and soil application with newly developed biopesticides on the soil population of *Fusarium oxysporum* f. sp. *ciceri* in the presence and absence of *Meloidogyne incognita*.

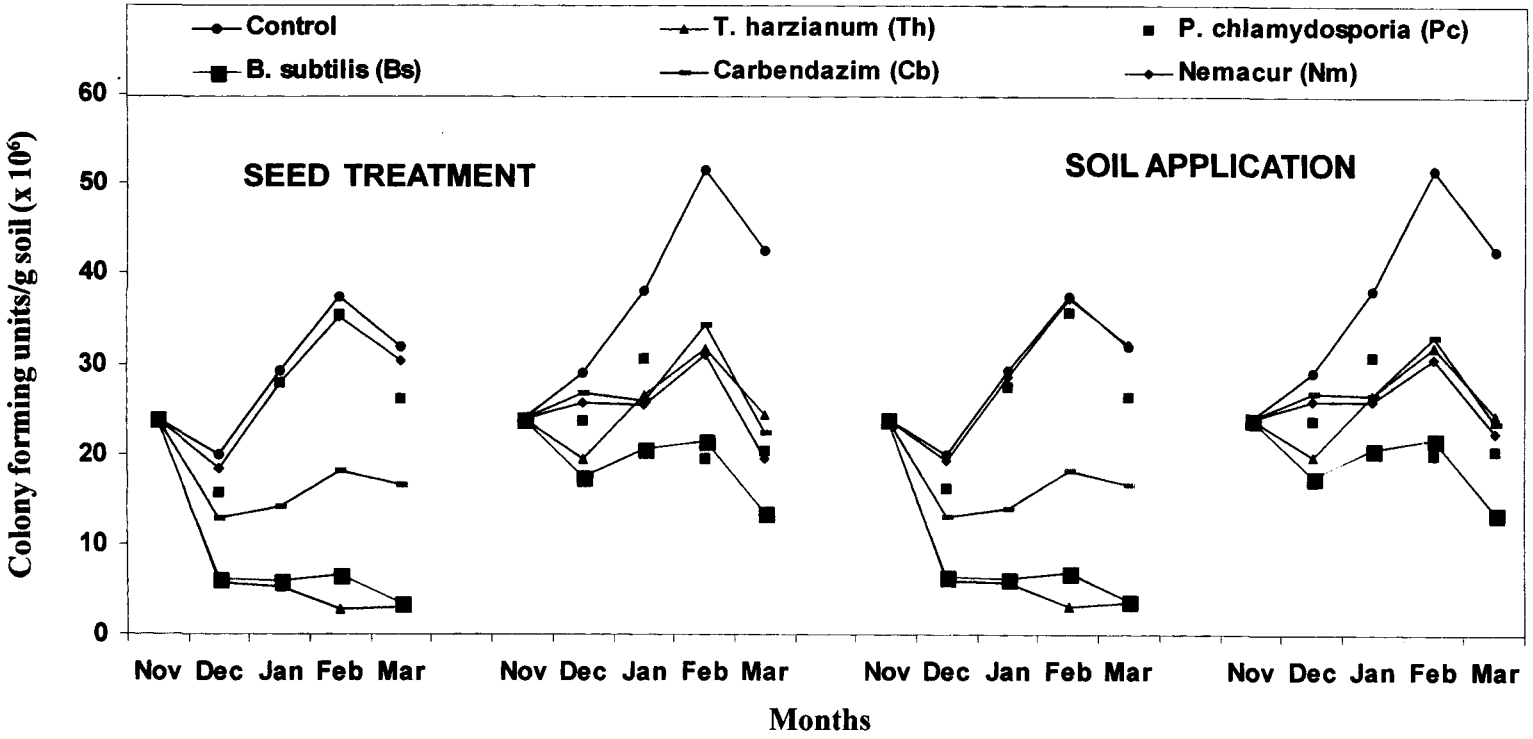
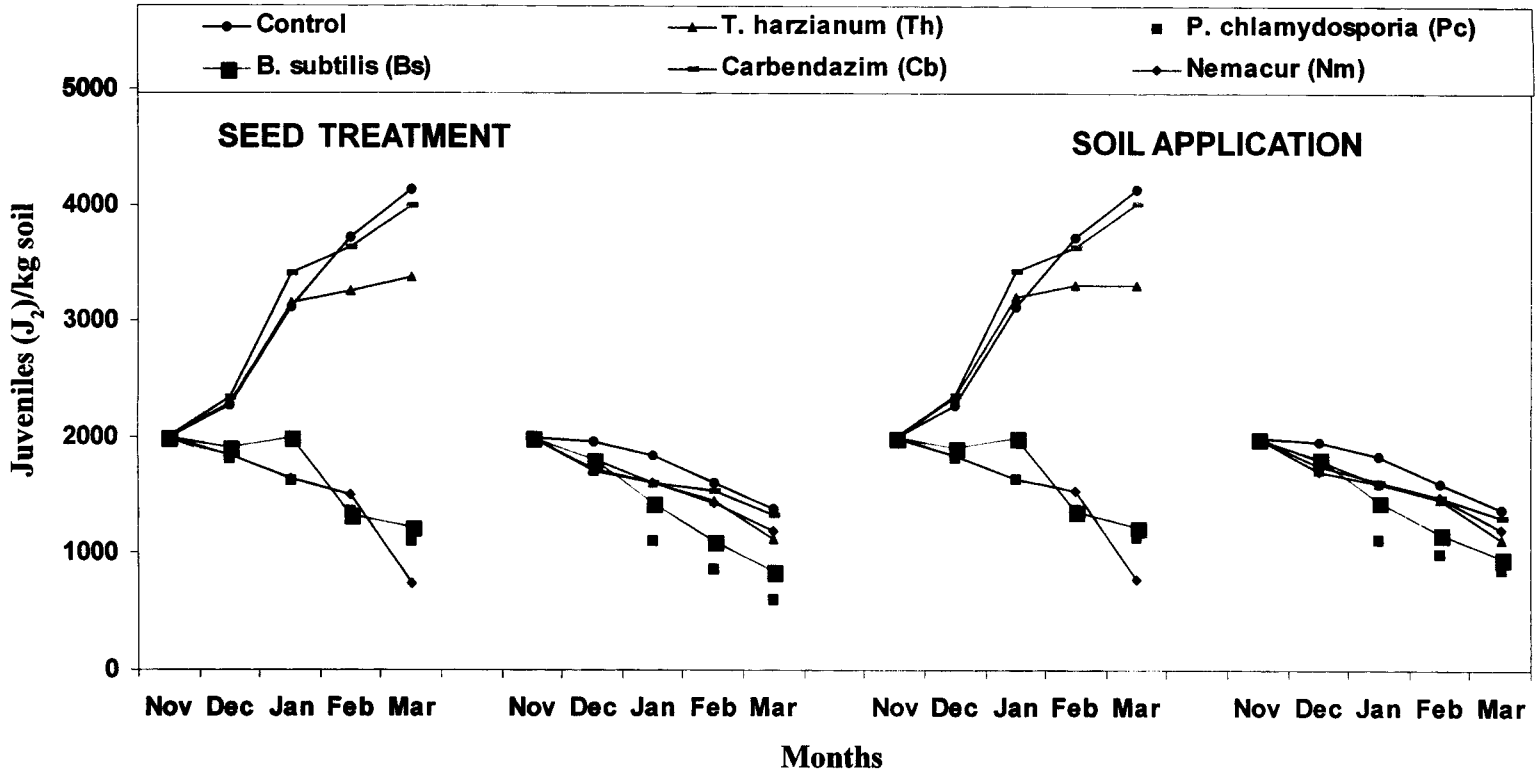


Figure 47. Effects of seed treatment and soil application with newly developed biopesticides on the soil population of *Meloidogyne incognita* in the presence and absence of *Fusarium oxysporum* f. sp. *ciceri.*



plots in comparison to the respective month control. Next in effectiveness was Biocure-X. Application of nemacur decreased the nematode population by 15-41% in comparison to the preplant population. Seed treatment of biopesticide in suppressing the nematode population was more or less similar to the soil application.

Rhizosphere population of the biocontrol agents

Trichoderma harzianum

In the plots not inoculated with pathogens but applied with Biowilt-X through soil or seed treatment, population of *T. harzianum* increased by 72-96% and 74-101%, respectively in comparison to the planting population (Fig. 48). In the presence of pathogens, the population further increased but to a varied extent in comparison to respective month controls. In the plots infested with wilt fungus and root-knot nematode, increase in the soil population of *T. harzianum* (seed treatment) was 42-69%, and 52-57%, respectively. In concomitantly inoculated plots, the population increased by 42-69% and 53-55% due to seed and soil application in comparison to planting population.

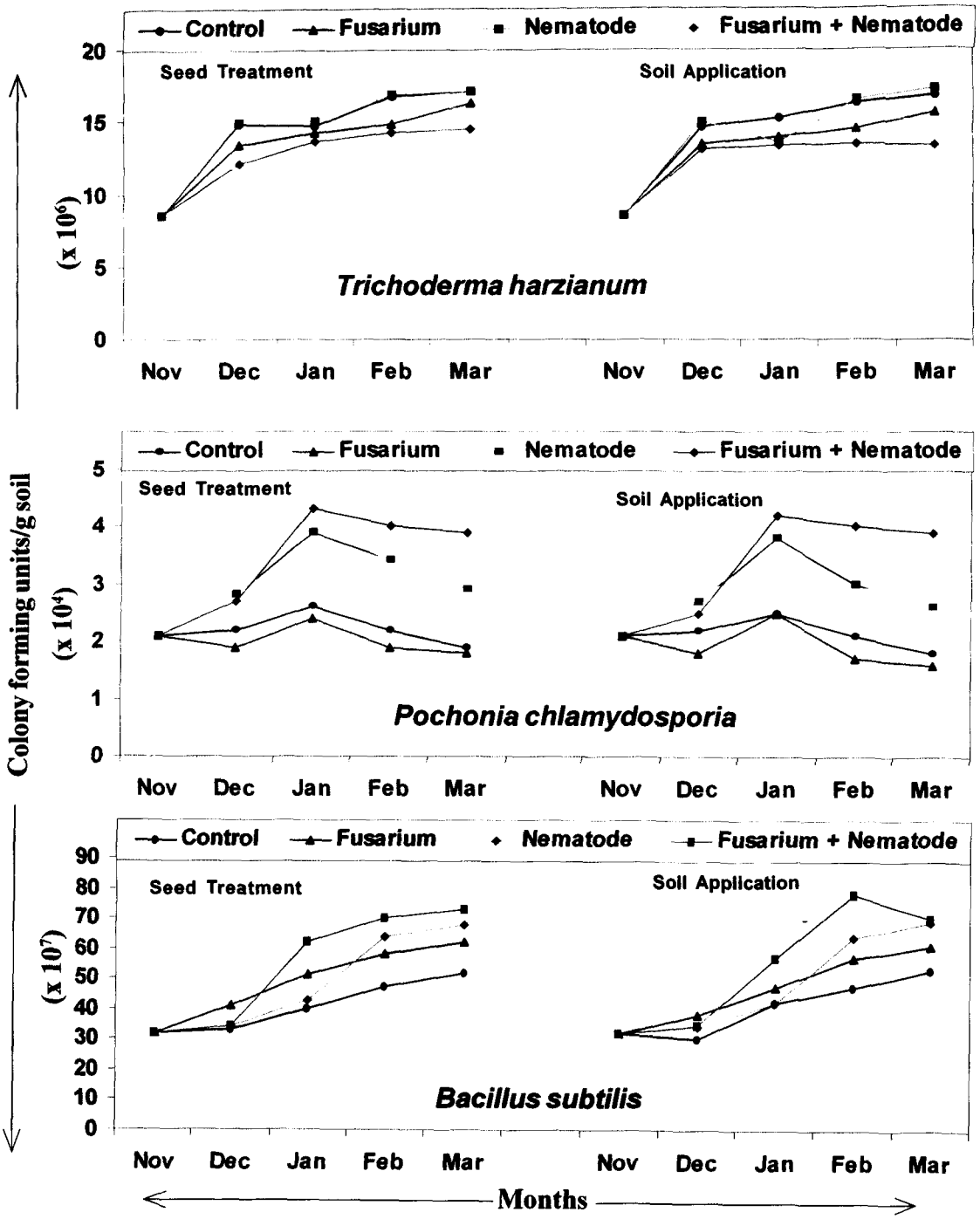
Pochonia chlamydosporia

Soil population of *P. chlamydosporia* in control plots increased significantly in January (Fig. 48). In the plots inoculated with the nematode and applied with Bionem-X, the soil population of the fungus increased by 27-54% in comparison to respective month control. In the plots infested with wilt fungus, population of *P. chlamydosporia* decreased with respect to planting/monthly population. In concomitantly inoculated plots, the population of *P. chlamydosporia* increased by 23-105% in comparison to monthly control and 28-104% in comparison to the planting population. In general, increase in the *P. chlamydosporia* population was relatively greater while applied on seeds than to the soil (Fig. 48).

Bacillus subtilis

Soil population of *B. subtilis* in uninoculated plots increased by 3-62% (seed treatment) and 31-66% (soil application) in comparison to planting population (Fig. 48). Soil population of *B. subtilis*, however, showed a decreasing trend in December. In the plots inoculated with the pathogens singly population of *B. subtilis* increased

Figure 48. Rhizosphere population of newly developed biopesticides in relation to single or concomitant inoculations with *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne incognita*.



significantly in comparison to respective month control. Increase in the bacterial population in plots infested with *F. oxysporum* f. sp. *ciceri* was greater than recorded in root-knot nematode infested plots. In concomitantly inoculated plots, the population of *B. subtilis* increased by 3-55%. Increase in the population of *B. subtilis* was relatively less when the biopesticide(s) was applied to soil (Fig. 48).

DISCUSSION

All the five biocontrol agents viz., *Trichoderma harzianum*, *T. virens*, *Pochonia chlamydosporia*, *Bacillus subtilis* and *Pseudomonas fluorescens* suppressed the wilt fungus *Fusarium oxysporum* f. sp. *ciceri* in preliminary *in-vitro* tests, the degree of antagonism, however, varied with the organism. The all six soil isolates of *T. harzianum* and *T. virens* overgrew the pathogen in the dual culture test, their suppressive effects also varied. Diversity in virulence and growth patterns within a *Trichoderma* species are well known to occur (Dennis and Webster, 1971a; Gao *et al.*, 2002). In the dual culture, hyphae of some isolates Th00, Th05 and Tv00 coiled around the hyphae of *F. oxysporum* f. sp. *ciceri* as soon as the two colonies when came in contact, whereas in other soil isolates, coiling did not occur until the *Trichoderma* hyphae had penetrated far into the host fungus colony. Other researchers have reported similar pattern of mycoparasitism by aggressive and less aggressive isolates to *T. harzianum* and *T. virens* on various pathogenic fungi (Lim and Teh, 1990; Nigam *et al.*, 1997; Gao *et al.*, 2001; Dubey *et al.*, 2007). The tested isolates also produced certain volatile compounds and suppressed the colonization by *F. oxysporum* f. sp. *ciceri.* The standard strains of *T. harzianum* (Th00) and *T. virens* (Tv00) caused greater reduction in the radial growth of *F. oxysporum* f. sp. *ciceri* than the isolate Th02 and Tv01. Effectiveness of the volatile compounds produced by *Trichoderma* spp. varied with the age of culture. Two day old cultures of both *T. harzianum* and *T. virens* caused greatest reduction in the radial growth of *F. oxysporum* f. sp. *ciceri* being greater with former species. With the age, the cultures lost ability to produce volatile metabolites and consequently the ability to suppress fungal growth decreasedv as evidenced by maximum colonization by the wilt fungus with 10 day old culture of *Trichoderma* species. In a classical study Dennis and Webster (1971c) investigated the production of volatile compounds by different isolates and species of *Trichoderma* and their effects on plant pathogenic fungi, *Fusarium, Sclerotium, Pythium, Rhizoctonia* etc, numerous researches have confirmed their findings (Padmodaya and Reddy, 1996; Kumar and Dubey, 2001; Dubey and Patel, 2001). The volatile compounds play an active role in the suppression of soil borne

plant pathogens (Lim and Teh, 1990; Angappan, 1992; Gao *et al.,* 2001; Gao *et al.*, 2002; Dubey *et al.*, 2007).

Suppressive effects of nonvolatile compounds (culture filtrates) of *Trichoderma* spp. on the radial growth of *F. oxysporum* f. sp. *ciceri* were found to be concentration dependent. Higher concentration of the culture filtrates (50, 75 and 100%) were more inhibitory than lower concentration (25%). Dennis and Webster (1971b) reported the production of nonvolatile compounds by *Trichoderma* spp. which were inhibitory to various pathogens. Gliotoxin or viridin were not produced by the mycoparasite, but other chloroform-soluble antibiotics, including trichodermin and peptide antibiotics were detected in culture filtrates. Other researchers have also reported the production of nonvolatile compounds by *Trichoderma* spp. and resulted inhibition in the radial growth of pathogens. *Trichoderma harzianum* isolate T_7 caused maximum growth inhibition of *Fusarium solani* f. sp. *pisi* causing collar rot of pea through production of non volatile substances (Kumar and Dubey, 2001). Growth of *Fusarium oxysporum* f. sp. *ciceri* was inhibited by the production of non volatile antibiotic substances with *Trichoderma* species (Dubey *et al.*, 2007).

The nonvolatile compound of the used biocontrol agents were also found nematicidal. The culture filtrates of *B. subtilis, P. fluorescens* and *P. chlamydosporia* suppressed egg hatch and induced mortality to the juveniles of *Meloidogyne incognita*. *Pochonia chlamydosporia* colonized egg masses of the nematode when they were incubated with the fungus in sterilized water, potato dextrose broth and decreased the egg hatch. The culture filtrates of *P. fluorescens* and *B. subtilis* also inhibited hatching and caused death to larvae but did not infect the egg masses or eggs. *In-vitro* antagonism through the culture filtrate of *B. subtilis* (Siddiqui and Mahmood, 1995), *P. fluorescens* (Siddiqui *et al.,* 2003; Siddiqui and Shaukat, 2004) and *P. chlamydosporia* (Saifullah, 1996 a, b, c) against plant parasitic nematodes is well documented, but degree of effectiveness varied with the isolate. *Bacillus subtilis* and *P. fluorescens* produce nematoxic metabolites that may have been involved in the nematode suppression (Gokte and Swarup, 1988; Khan and Akram, 2000; Siddiqui and Eteshamul-Haque, 2001). *Bacillus subtilis* produces the metabolites bacillomycin (Bessen and Michel, 1984; Brannen, 1995), iturin, surfactin (Asaka and Shoda, 1996a),

and agrocin (Kim *et al.*, 1997a). Phloroglucinol (Howell and Stipanovic, 1984), pyrrolnitrin (Leyns *et al.*, 1990) and phenazin (Gurusiddaiah *et al.*, 1986) are produced by *P. fluorescens*. *Pochonia chlamydosporia* is an established biocontrol agent of root knot nematodes, the fungus parasitizes nematodes and their eggs (Khan *et al.*, 2001; Stirling, 1991). In the present study, isolates of *P. chlamydosporia*, *P. fluorescens* and *B. subtilis* induced varied degree of inhibition in hatching and mortality to juveniles of *Meloidogyne incognita*.

The integration of biocontrol agents along with fungicides for effective management of soil borne diseases is present day's need. The tolerant strains of biocontrol agents against pesticides need to be identified and integrated in managing the diseases. Attempts have been made to develop tolerant strains against carbendazim (Yang and Zhao, 1996) and some other fungicides (Pecchia, 1994; Cuevas *et al.*, 1994; Migheli *et al.*, 1995; Mrinalini and Lalithakumari, 1998). In the present study, compatibility of *Trichoderma harzianum, T. virens, P. chlamydosporia, B. subtilis* and *P. fluorescens* with two systemic and four nonsystemic fungicides was examined. The concentrations of 60 µg carbendazim/ml, 1050 µg metalaxyl/ml, 1500 µg/ml, 160 µg captan/ml and 225 µg mancozeb/ml seem to be safe tolerance limit (ED_{50}) for *T. harzianum* whereas the corresponding values for *T. virens* were 40 µg carbendazim/ml, 1000 µg metalaxyl/ml, 1720 µg nemacur/ml, 125 µg captan/ml and 177 µg mancozeb/ml. The ED_{90} values of thiram for growth of *T. harzianum* and *T. virens* were 150 and 95 µg/ml medium. Whereas, 25 and 9 µg/ml concentrations seem to be safe tolerance limit (ED_{50}) for *T. harzianum* and *T. virens,* respectively. Similar results have been obtained by other workers. Sharma *et al.* (2001) found 0.1% metalaxyl and 0.0065% carbendazim as safe tolerance limit (ED_{50}) for *T. harzianum.* More or less similar values have been were obtained for carbendazim and benomyl (Papavizas *et al.*, 1982; Jayaraj and Radhakrishnan, 1997; Viji *et al.*, 1997) and for metalaxyl (Mukhopadhyay *et al.*, 1986; Mukherjee *et al.*, 1989). Different workers have reported chlorothalonil, captan and captafol as tolerant for *T. harzianum* even at higher concentrations upto 2000 mg/ml in spore germination tests (Abdel-Moity *et al.*, 1982; Papavizas *et al.*, 1982; Mishra *et al.*, 2004). More researches are needed on the integration of *Trichoderma* spp. with chemical approaches to improve effectiveness of the disease

control module. *P. chlamydosporia* showed less tolerance to the five fungicides tested than *Trichoderma* spp., whereas the biocontrol bacteria demonstrated the tolerance greater than the fungi. Among the bacteria, *P. fluorescens* was found to be more compatible with fungicides than *B. subtilis,* the MTC for the former being 2500 µg Thiram/ml, 1600 µg mancozeb/ml and 50, 000 µg/ml for captan and carbendazim. Researchers have shown that some bacteria can use pesticides as nutrients and hence can tolerate higher concentrations of chemicals (Kishore and Jacob, 1987; Aislabie and Jones, 1995).

In vitro studies were also carried out to study the fermentation, biochemical characteristics and phosphate solubilization of the isolates of *B. subtilis* and *P. fluorescens.* All the isolates differed in the fermentation behaviour and biochemical properties. Similar variations in biochemical and fermentation behaviour of soil isolates of *B. subtilis* and *P. fluorescens* have been reported by other researchers (Sattar and Gaur, 1989; Gaind and Gaur, 1991; Mahmood, 1999). All six strains each of *P. fluorescens* and *B. subtilis* solubilized phosphorus evidenced by the zone of solubilization on agar plates; diameter of the zone was wider with *P. fluorescens* than *B. subtilis* and varied with the isolate. *P. fluorescens* and *B. subtilis* are well known for phosphate solubilization (Gaur, 1990; Dave and Patel, 1999). Production of organic acids by the bacteria is one of the important determinants of phosphorus solubilization (Gaur, 1990; Satpul and Kapoor, 1992; Singal *et al.,* 1994; Illmer and Schinners, 1995; Dave and Patel, 1999). In the present study an inverse relation was observed between pH value and phosphate solubilization *in vitro*. It is an established fact that *P. fluorescens* and *Bacillus subtilis* produce growth promoting substances (phytohormones) like indole acetic acid (IAA), gibberellic acid etc. (Pal *et al.*, 2001; Gracia de Salamone *et al.,* 2001). However, amount of IAA production depends on the species and strain. In the present study *P. fluorescens* and *B. subtilis* produced IAA in Luria Bertani broth (LBB) supplemented with tryptophan. Other researchers have also detected IAA production by *P. fluorescens* and *B. subtilis* in the supplemented LBB (Oberhansli *et al.,* 1991; Glickman *et al.,* 1998; Kawaguchi and Syono, 1996).

The wilt fungus, *F. oxysporum* f. sp. *ciceri* at the inoculum level of 2 g/kg soil caused considerable wilting in chickpea and suppressed the growth and yield of plants

grown in the pots or field. The wilt symptoms on chickpea can appear 3 to 4 weeks after sowing. The infected seedlings may collapse and lie on the ground. The seedlings may also remain erect but appear dull green in colour. Adult plants show typical wilt symptoms of drooping of petioles, rachis and leaflets (Gurha *et al.*, 2003). In the present study, chickpea responded similarly to the inoculation with the fungus and developed typical symptoms of the disease both at the seedling and adult stage. The first sign of the disease was mild chlorosis and stunted growth that appeared at seedling stage. Some of the stunted seedlings succumbed to the infection. Under field conditions, recognizable wilt symptoms developed when plants were 6-8 weeks old. The seedlings, which escaped early infection exhibit stunted growth and leaf chlorosis at one month age. At a later stage, leaves/branches wilted, drooped or dried. The pathogen infects the root system by penetrating the epidermis, cortex and finds its way into xylem vessels where it colonizes extensively, producing conidia. As a result black streaks gradually develops in xylem tissue whereas brown to black bands appear on the stem surface of partially wilted plants which extend upward from the base. When the bark of such bands is peeled off, browning or blackening of the wood beneath can be seen (Dubey and Singh, 2004). In the present study, transverse sections of root and stem revealed the presence of the fungus in xylem tissue. The fungus also colonized on PDA by inoculating the surface sterilized root and stem pieces of infected chickpea plants. The isolated fungus was then reinoculated in the chickpea plants to establish its pathogenicity.

The used chickpea cultivar BG 256 was found to be highly susceptible to wilt and exhibited 24-38% yield loss. The annual monetary loss in chickpea production due to the wilt has been estimated to be 12 million rupees in Pakistan (Sattar *et al.*, 1953). In the present study, monetary loss of Rs. 13, 000/ha was estimated due to wilt in chickpea. Application of biocontrol agents either through soil application or seed treatment considerably decreased the pathogenic effect of the wilt fungus in pot experiments. As a result plant growth and yield of chickpea increased significantly. Seed treatment with *T. harzianum* checked the wilt incidence by 60%, whereas its soil application resulted to a 58% control of the disease. *T. virens* was also equally effective against the disease. *Trichoderma* spp. are the established antagonists of soil borne

fungal pathogens (Papavizas, 1985). The satisfactory control of the wilt indicates that the used strain of *T. harzianum* and *T. virens* (strain) suppressed the pathogen effectively.

The three biopesticides namely, Biowilt-X (*T. harzianum*), Bionem-X (*P. chlamydosporia*) and Biocure-X (*B. subtilis*) prepared on fly ash based carrier contained 10^{8-10} CFUs of biocontrol fungi and 10^{12-13} CFUs of biocontrol bacteria/g formulations. The shelf life tests revealed highest CFU load during 6-12 weeks of inoculation. Increase in CFU count during storage indicates that the biocontrol agents utilized the nutrients present in fly ash-molasses mixture. Bacteria and fungi can degrade and utilize the essential elements of fly ash (Fakoussa and Hofrichter, 1999; Schmidt and Noack, 2000) and can utilize the sugars present in molasses (Maneerat, 2005). Molasses contains 48-56% total sugar (mainly sucrose), 9-12% non-sugar organic matter, 2-4% protein, 1.5-5% potassium, 0.4-0.8% calcium, 0.06% magnesium, 0.6-2.0% phophorus, 1.0-3.0 mg biotin/kg, 15-55 mg pantothenic acid/kg, 2500-6000 mg inositol/kg and 1.8 mg thiamine/kg (Curtin, 1983; Makkar and Cameotra, 1997). These nutrients can serve as a good source of carbohydrates for sustenance and multiplication of microorganisms (Patel and Desai,1997; Maneerat, 2005). Because of low cost of ingredients used to prepare present commercial formulation, cost of the biopesticides came much lower than their contemporaries available in Indian market. The shelf life test has revealed that the present biopesticides contained CFU load of biocontrol agents on or above the standard level of 10^{8-9}/g formulation (Tilak, 1993). The highest CFU counts were recorded at room temperature (22-37°C) or 25°C during 6-14 weeks of storage. These temperatures and durations well suit Indian condition as ambient temperature normally remains 20-35°C except during January-February and May-July. A six week period is sufficient for transportation and distribution of the biopesticides to local pesticide dealers. and another 3-6 weeks for the procurement of the biopesticide by a farmer and its application in the field. Hence the total duration from manufacturing to application lies within the period of higher CFU load as determined by the shelf life test. It, is, therefore well expected that when the biopesticide will reach in the field it will contain the standard CFU load of biocotnrol agents.

Effectiveness of the newly prepared biopesticides was evaluated against the target diseases on chickpea in small plots under field condition. Inoculation with

F. oxysporum f. sp. *ciceri* resulted to 60% wilt incidence with an average severity of 3.8 on 0-5 scale. Application of the biocontrol agents provided varied disease control. Greatest decrease in the wilt was recorded with the application of *T. harzianum* and *B. subtilis* applied through crude formulation (bagasse-soil molasses mixture). Application of their commercial formulations, Biowilt-X and Biocure-X checked the wilt incidence by 60 & 55% and the wilt severity by 63 & 51% over control. Effect of carbendazim was almost equal to Biowilt-X. Other researchers have also shown that if biofungicides contain standard CFUs of efficient strains of the antagonist they can provide disease control equal to fungicides (Khan, 2005).

Root-knot nematodes are sedentary obligate endoparasites and they induce certain structural, physiological and biochemical changes in the host plant (Haung, 1985; Hussy, 1989). Second stage juveniles enter into young lateral roots, and after getting a suitable site for feeding become sedentary with their heads inserted in vascular tissue and body in the cortical region of the root. As a result of nematode pathogenesis, a few cells around the head region preferably in primary phloem are transformed into multinucleate giant cells. These cells are permanent feeding sites of the nematodes. Giant cells, which act as transfer cells or nurse cells provide nutrition to the sedentary females throughout the life span. Whereas cells around the female body become hyperplastic dividing repeatedly by mitosis resulting to enlargement of the tissue which is commonly called as gall or knot (Bird, 1972). Galled roots become short, thick and deshaped. Root growth suppression disturbs absorption capacity of the root resulting to the appearance of water stress symptoms in foliage, especially during periods of moisture stress and high temperature (Wilcox-Lee and Loria, 1987). Photosynthesis decreases but respiration rate increases (Wilcox-Lee and Lorea, 1987) accompanied by greater allocation of photosynthates to roots particularly infected tissue and the giant cells (Wallace, 1987). Cumulatively the infection leads to poor growth and reduced yield of the host. Infected plants show nutrient deficiency symptoms which can be recognized in field as patch of plants showing stunted growth with pale green foliage. In the present study the chickpea cv. BG 256 inoculated with 2000 J_2/kg soil showed poor shoot growth and mild leaf chlorosis, and discernible galls formed on the roots. The galls were, however, small in size. On an average 81 galls and 72 egg masses/root system

were formed in the pot trial whereas in field grown plants 75 galls and 67 egg masses developed per root system. Application of biocontrol agents or their commercial formulation (biopesticides) suppressed the gall formation and egg mass production. Application of *P. chlamydosporia* and *B. subtilis* or their biopesticides caused the highest decrease in the number of galls and egg masses per root system followed by nemacur.

Pochonia chlamydosporia is an important parasite of root-knot nematodes (Stirling, 1991). The fungus is also known to produce some exoenzymes that help in disintegration of egg shell (Seggers *et al.*, 1996). The used strain of the antagonist demonstrated strong nematicidal activity *in vitro* evidenced by 100% mortality in the juveniles of *M. incognita* due to 50, 75 and 100% culture filtrate. When the egg masses were incubated in the suspension of *P. chlamydosporia*, the fungus colonized them and inhibited the egg hatch to a great extent. Strains of *P. chlamydosporia* and *T. harzianum* have been found to parasitize the eggs of *G. rostochiensis, G. pallida* and *Panagrellus redivivus* leading to significant decline in the respective soil populations (Saifullah, 1996a, b, c; Viaene and Abawi, 2000).

Treatments with *B. subtilis* suppressed the galls, egg masses and soil population of *M. incognita* but it was less than *Pochonia chlamydosporia* in both pot or field trial. *B. subtilis* is not a parasite of plant nematodes, but the bacterium may have suppressed nematode infection through other mode of action. The suppression of nematode infection may result through (i) production of antibiotics such as bacillomycin (Schoonbeck *et al.*, 2002), iturin (Burkhead *et al.*, 1994), and siderophores (Leong, 1986) which may affect egg hatch and oriented movement of larvae.

Pant and Pandey (2002) have reported a significant reduction in the galling caused by *M. incognita* when *T. harzianum* was applied. However, in the present study a discernible antagonism by the strains of *T. harzianum* or *T. virens* on *M. incognita* was not noticed both in pots and field. *Trichoderma* spp. are active colonizers in soil and preferably grow on organic material. The fungus may have colonized larvae and subsequently adults, eggs or egg masses, and resulted to significant decline in galls and soil population of *M. incognita* in the pots. This was probably due to the organically rich soil and the confined space in pots which may have supported greater multiplication

of the antagonist. *Trichoderma* spp. produce certain enzymes/metabolites such as gliotoxin (Weindling, 1941), hydrolytic enzymes (Schermbock *et al.*, 1994), endochitinase, B- glycosidase, B-glyconase and chitobiosidase (Lorito *et al.*, 1993), which may have been involved in the antagonism against root-knot nematodes. Sharon *et al.* (2001) have reported a significant decrease in galling with the application of *Trichoderma* spp.

Soil population of the pathogens significantly decreased due to application of biocontrol agents or pesticides. Carbendazim (Nene and Thapliyal, 1993) and nemacur (Johnson, 1985) and are efficacious pesticides and their application usually result to considerable decline in the severity of wilt and root-knot and soil populations of *Fusarium* and *Meloidogyne* spp., respectively (Neophytou *et al.*, 2002; Saleh *et al.*, 2002; Agrawal *et al.*, 2003). Greatest decrease in the soil population of *Meloidogyne incognita* occurred with the application of *P. chlamydosporia* followed by *B. subtilis*. .The *P. chlamydosporia* and the other biocontrol agents used may have suppressed the nematode population with the actions as explained for decrease in galls and egg masses. An inverse relationship was recorded between the soil populations of a pathogen and biocontrol agent. Soil population of the biocontrol agents increased proportionately with the decrease in disease severity and pathogen population. Greatest increase in the population of biocontrol agents was recorded for *T. harzianum* in the wilt fungus infested plots. The antagonist, *T. harzianum* parasitizes *F. oxysporum f. sp. ciceri* (Singh *et al.*, 2002) and draw nutrition from mycelium to grow and sporulate (Pandey and Upadhayay, 2000). Likewise, *P. chlamydosporia* is a parasite of root-knot nematodes (Kerry, 2000) and its population increased in the pots/plots inoculated with *M. incognita*. The fungus multiplies efficiently on eggs and egg masses of *Meloidogyne* species (Stirling, 1991; Khan *et al.*, 2005a). The fungus population was greater in the plots that had nematode alone than together with *F. oxysporum f. sp. ciceri*. The wilt fungus is well documented to utilize feeding sites of root-knot nematodes (Francl and Wheeler, 1993), as a result the nematode development and egg mass production are suppressed. Due to this less availability of preferred substrate (egg masses) the *P. chlamydosporia* population could not increase as much as in the plots infested with *M. incognita* alone. *B. subtilis* is not a parasite of *M. incognita*, but greater increase in its population in the nematode infested

soil indicates that presence of pathogens facilitated the bacterial multiplication. This may have occurred through host, especially the root exudation. Root exudates contain carbohydrates and other nutrients which serve as energy source for multiplication of rhizobacteria including *B. subtilis* (Scher *et al.*, 1988). Penetration and/or infection by *M. incognita* cause greater root exudation and leakage of cell contents from roots resulting to 5-12% greater increase in the soil population of the bacteria in the nematode infected soils.

Synergistic interaction between root-knot nematodes (*Meloidogyne* spp.) and wilt inducing fungi (*Fusarium* spp.) is well established on a number of crops including legumes like, alfalfa (Griffin, 1986), cowpea (Thomason *et al.*, 1959), pea (Davis and Jenkins, 1963), beans (Ribero and Ferraz, 1984), mungbean (Khan *et al.*, 2002), chickpea and pigeonpea (Khan, 2005). The interaction between these two pathogens has been found to be generally synergistic (Francl and Wheeler, 1993). In such interactions the fusarial wilt becomes severe in presence of the nematodes resulting to significantly greater crop damage. In the present study also, *M. incognita* and *F. oxysporum* f. sp. *ciceri* interacted synergistically and caused greater suppression in plant growth and yield parameters of chickpea cv. BG 256. Severity of wilt symptoms was enhanced when both the pathogens were present together in comparison to *F. oxysporum* f. sp. *ciceri* alone. Root-knot nematode infection leads to alteration in root exudation of the infected plants. Root exudates of nematode infected plants contain greater concentration of Ca, Mg, Na, K, Fe and Cu, and during first fourteen days of infection, carbohydrates are the major organic constituents of root exudates and nitrogenous compounds predominate afterwards (Van Gundy *et al.*, 1977). According to Powell (1971) and Webster (1985) root-knot nematode infection predisposes the host plant to wilt fungus. During development of giant cells, infected roots of host plant exhibit decreased concentration of cellulose and lignin, and considerable increase in the amino acids, hemicelluloses, lipids, minerals, nucleotides, organic acids, proteins, DNA and RNA (Khan, 1993). These biochemical changes enrich the medium, which is the cause for rapid growth and colonization of the wilt fungus (Francl and Wheeler, 1993). Giant cells being rich nutritionally, serve a good site for the colonization of the fungus. The giant cells, located in the vascular tissue act as a launching pad for the fungus for spread

in xylem tissue and also to transport toxins. Wilt fungus produce fusaric acid and other toxins that contribute in the development of wilting symptoms (Bell and Mace, 1981; Glick, 1995).

The concomitant inoculation with *M. incognita* and *F. oxysporum* f. sp. *ciceri* in the present study, exacerbated the wilt symptoms but severity of nematode symptoms (galling) significantly decreased compared to *M. incognita* inoculated control. *Fusarium* spp. have shown strong affinity to feeding sites of sedentary endoparasites. The giant cells formed by *Meloidogyne* spp. are rapidly invaded by the wilt fungus utilizing its contents (Webster, 1985) as a result developing nematode females starve to death. In addition, the metabolites produced by *F. oxysporum f. sp. ciceri* may suppress hatching of eggs and induce mortality to larvae (Ciancio *et al.*, 1988). The sedentary endoparasites such as *Meloidogyne* spp. show decreased populations in soils infested with *Fusarium* or *Verticillium* (Hasan, 1989; Fazal *et al.*, 1994).

Nodule formation on chickpea was quiet good and it further increased due to application of biocontrol agents. *B. subtilis* significantly promoted the nodulation. Infection by *F. oxysporum f. sp. ciceri* and *M. incognita* singly or concomitantly decreased the number of functional and total nodules/root system in comparison to the control. Decrease in nodulation by concomitant inoculation was significantly greater than the individual effects of the two pathogens. Suppressive effects of *Fusarium* spp. on root nodules has been observed in a number of studies (Twng-Wah and Howard, 1969; Sawada, 1982 and 1983), but the mechanism involved is not properly understood. It looks plausible that the fungus infected roots due to physiological and/or structural modifications become unsuitable for the infection by the rhizobium and development of root nodules and vice versa. The suppression may also be due to competition between the two microorganisms at initial stage of the infection. Fusaric acid produced by the *Fusarium* spp. (Toyoda and Utsumi, 1991) may also be involved in inhibiting the rhizobium. Wilt causing fusaria are known to cause less infection on nodulated roots than non-nodulated roots (Zombolim and Schenk, 1984).

Antagonistic interaction between root nodule bacterium and root-knot nematode was recorded resulting to decrease in the nodulation. Mutual antagonism between *Meloidogyne* and *Rhizobium* evidenced by decreased number of nodules has been well

documented (Huang and Barker, 1983; Verdejo *et al.*, 1988). Various explanations including competition for space and nutrition between the two organisms have been offered for this kind of mutual inhibitory effects (Taha, 1993). The suppression of root nodulation in chickpea may have occurred due to nutritional interference, particularly carbohydrates or physiological changes brought about by the nematode infection and/ or competition for infection site (Taha, 1993). Number of functional nodules were found to be decreased but non functional nodules increased significantly as a result of infection by *M. incognita*. This may be due to invasion of nodules by the nematode and causing histological changes in the nodular tissue (Taha and Raski, 1969; Barker and Hussey, 1976; Khan *et al.*, 2002) thereby rendering them nonfunctional.

RAPD analysis based on six, five and four random primers for *Pochonia chlamydosporia, Trichoderma harzianum* and *Bacillus subtilis* respectively revealed a great deal of intra-specific similarity amongst isolates of *T. harzianum*, *P. chlamydosporia* and *B. subtilis* examined. This is in accordance with several researchers who used RAPD-PCR to differentiate isolates of *Trichoderma* sp. (Abbassi *et al.*, 1999), *P. chlamydosporia* (Mauchline *et al.*, 2004) and *B. subtilis* (Matarante *et al.*, 2004). Recent researches have however indicated that RAPD-PCR is not a reliable test to differentiate isolates of a species. However within the available limited infra-structure, other most reliable test such RFLP could not be done.

In the present study the used strain of *B. subtilis* was found to be an efficient plant growth promoter. Its application resulted to significantly greater dry matter and yield of chickpea. *Bacillus subtilis* produces phytohormones (Archipora *et al.*, 2005), solubilizes minerals and produces siderophores that can solubilize and sequester iron from the soil and provide it to plants cells (Leong, 1986). Application of biocontrol agents or their commercial formulations checked the suppressive effect of the pathogens on chickpea leading to significant increase in the dry matter production and yield. Chickpea plants infected with *M. incognita* and *F. oxysporum* f. sp. *ciceri* singly or concomitantly produced significantly greater dry matter and grains with *B. subtilis* treatments compared to other treatments. The treatment with *B. subtilis* enhanced the plant growth and yield greater than other biocontrol agents used. Although decrease in the disease severity with the bacterium was less than other treatments but yield

enhancement was greater. Apparently *B. subtilis* may have acted through two ways. Yield enhancement partly occurred due to plant growth promotion and partly due to disease suppression. *P. fluorescens* and *Trichoderma* spp. are also reported to solubilize phosphorus (Kole and Hajra, 1997; Sharma, 2003) and produce antibiotics (Brannen, 1995; Haggag and Mohamed, 2002) but in the present study *B. subtilis* was found much more efficient. Some other researches have also indicated effectiveness of *B. subtilis* greater than *P. fluorescens* in enhancing the plant growth and yield (Landa *et al.*, 2004). Treatments with nemacur or carbendazim checked the disease much greater than *B. subtilis* but yield enhancement in chickpea was less than the bacterial treatment.

Overall seed treatment with biocontrol agents was relatively more effective than soil application. The amount of biocontrol agents added to a microplot through soil application (40 g/microplot) was much greater than that carried by the seeds (5 g/kg seed). But with soil application, the CFU's dispersed in a greater area giving rise to a much smaller CFU count/unit, whereas with seed treatment the microorganisms remained concentrated on or around the seed and later on in the root zone. The germinating seeds attract rhizobacteria (Scher *et al.*, 1985) and are rapidly colonized due to profuse exudation of a wide range of amino acids, carbohydrates, organic acids (Hayman, 1969; Lynch, 1978). The fungi and bacterial biocontrol thus received greater nutrients through exudates of germinating seeds to full root growth and also had faced less competition and exerted more because of their preoccupation and/or aggregation in a limited area in close vicinity of roots, as a result the applied strains multiplied with a greater pace evidenced by the higher CFUs/g soil observed during crop growth in the microplots which received biocontrol agents through seed treatment. Because of greater CFU/g soil, phosphate solubilization, hormone production and/or pathogen suppression by the biocontrol agents would have been greater in the root zone and reflected into better disease control, plant growth and yield of chickpea.

The present study had demonstrated that the three biopesticides namely, Biowilt-X (*Trichoderma harzianum*), Bionem-X (*Pochonia chlamydosporia*) and Biocure-X (*Bacillus subtilis*) developed are superior than their contemporaries available in the market with regard to effectiveness and cost. The formulations have a CFU load in the range of 10^{7-9} (biocontrol fungi) and 10^{12-13} (biocontrol bacteria), which is greater than

other biopesticides. The bioagents applied through the commercial formulation established in the soil and hence can confer a longer crop protection. Application of Biowilt-X and Bionem-X biopesticides @ 5g/kg seed satisfactorily managed fusarial wilt and root knot diseases of chickpea, and gave a profit of Rs 4000/ha against wilt and Rs 1750/ha against root-knot. Treatment with Biocure-X (*Bacillus subtilis*) @ 5g/kg seed effectively controlled the fusarial wilt, root knot and wilt disease complex of chickpea with a profit of Rs 7750/ha.

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APPENDICES

Appendix-1

Potato Dextrose Agar

Potato	200 g
Dextrose	20 g
Agar	15 g
Water	1 l

Appendix-2

Trichoderma Specific Medium (TSM)

Glucose	3.0 g
KCl	0.15 g
$NH4NO_3$	1.0 g
$Mg\ SO_4 7H_2O$	0.2 g
K_2HPO4	0.9 g
Agar	15 g
Water	1 l

After autoclaving add 250 mg chloramphenicol, 300 mg fenaminosulf, 200 mg quintozene and 150 mg Rose Bengal + 20 mg captan to eliminate *Fusarium*.

Appendix-3

Pochonia chlamydosporia Semi-Selective Medium

Corn Meal agar	17 g
Streptomycin sulphate	50 mg
Rose Bengal	75 mg
Aureomycin	50 mg
NaCl	17.5 g
Chloramphenicol	50 mg
Carbendazim	37.5 mg
Triton X-100	3 ml
Thiabendazole	37.5 mg
Water	1l

Appendix-4

Bacillus subtilis Isolation Agar

Peptic digest of animal tissue	6.0 g
Casein enzymic hydrolysate	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1l

Appendix 5
King's B Medium with supplements

Proteose peptone	20 g
K_2HPO4	2.5 g
$Mg\ SO_4 7H_2O$	6 g
Glycerol	15 ml
Agar	15 g
Water	1l

Novobiocin 45 mg; penicillin G 44.9 mg (75, 000 units); cycloheximide (actidione) 75 mg were mixed in 3 ml of 95% ethanol. Diluted with 50 ml of sterile distilled water and added to the one litre of the above medium. 20 mg tetracycline was also added to make the medium specific for the used *Pseudomonas fluorescens* strain.

Appendix 6
Phenol-red nutrient broth or Fermentation broth

Proteose peptone	10 g
NaCl	5 g
Phenolred	0.018 g
Water	1l

Five grams (5.0 g) each of glucose, lactose, fructose, sucrose and nutrient agar maltose was added to the medium separately.

Appendix 7
Peptone Broth

4% aqueous solution of peptone.

Appendix 8
Nutrient agar

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Water	1 l

Appendix 9
Solidified Simmon's Citrate Agar

$(NH_4)\ H_2PO4$	1.0 g
K_2HPO4	1.0 g
NaCl	5.0 g
Sodium citrate	2.0 g
$Mg\ SO_4$	0.2 g
Bromothymole blue	0.08 g
Agar	15 g
Water	1 l
pH	6.9

Appendix 10
Nutrient gelatin

peptone	5.0 g
Beef extract	3.0 g
Gelatin	120 g
pH	6.8

Appendix 11
Kovac's Reagent

p-dimethylaminobenzalde	50.0 g
Amylalcohol	750 ml
Hydrochloric acid(concentrated)	250 ml
Water	1000 ml

Appendix 12
Methyl red Voges Proskauer broth

Peptone	7.0 g
Dextrose	5.0 g
Potassium phosphate	5.0 g
Water	1 l

Appendix 13
Nitrate broth

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	5.0 g
Water	1 l

Solution A: Sulfanilic acid

sulfanilic acid	8.0 g
5 N acetic acid	1000.0 ml

Solution B : α–napthyl amine

Dimethyl-1-napthylamine	5.0 g
5 N acetic acid	1000 ml

Appendix 14
Triple sugar iron agar

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Proteose peptone	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous sulphate	0.2 g
Sodium chloride	5.0 g
pH	7.4
Water	1 l

Appendix 15
Barrit's reagent (g/100ml)

Solution A	
Alpha naphthol	5.0
Ethanol(absolute)	95 ml
Solution B	
Creatinin	0.3
Potassium hydroxide	40.1

Appendix 16
Trypticase-soy Agar

Trypticase	17.0 g
Phytone	3.0 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Glucose	2.5 g
Agar	15 g
Water	1l
pH	7.3

Appendix 17
Luria Bertani Broth

Bacto Tryptone	10 g
NaCl	5 g
Yeast Extract	5 g
Water	1l
pH	7.0

Appendix 18
Reagent $FeCl_3HClO_4$

1ml of 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$

Appendix 19
Pikovskaya's Medium

Glucose	10.0 g
$Ca_3(PO_4)_2$	5.0 g
$(NH_4)_2SO_4$	0.5 g
NaCl	0.2 g
$MgSO_4.7H_2O$	0.1 g
KCl	0.2 g
Yeast extract	0.5 g
$MnSO_4$	Trace
$FeSO_4.7H_2O$	Trace
Agar	15.0 g
pH	7.0

Appendix 20
Chloromolybdic acid reagent

15g of ammonium molybdate was dissolved in about 400 ml of warm distilled water to which 342 ml of 12 N HCl was added slowly and finally the volume was made one litre with distilled water.

Appendix 21
Chlorostannous acid reagent

The reagent was prepared by dissolving 2.5g of $SnCl_2.2H_2O$ crystals in 10 ml concentrated HCl and finally the volume was made 100 ml with distilled water.

Appendix 22
Standard phosphorus solution (100ppm)

0.4390gm of dried KH_2PO_4 was dissolved in 400 ml distilled water. 0.25 ml of 7N H_2SO_4 was added to it and the volume was made upto one litre.

Standard phosphorus solution (2ppm)

2 ml of 100 ppm standard solution was diluted to 100 ml distilled water.

Appendix 23
Fusarium Specific Medium/Nash and Snyder Medium/PCNB Agar

Peptone	5.0 g
Mg $SO_4 7H_2O$	0.5 g
KH_2PO4	1.0 g
Agar	20 g
Water	1 l

After autoclaving, the medium was cooled to 45°C and supplemented with 300 mg streptomycin and 1 g PCNB (75% WP).

Appendix 24

Climatic conditions that prevailed during cropping season 2004-05

Months	Temperature			Relative humidity			Rainfall
	Max.	Min.	Mean	Max.	Min.	Mean	
Nov	32.78	17.62	25.20	73.91	42.98	58.45	15.6
	30.72	16.64	23.68	75.92	47.94	61.93	0.0
	29.96	16.40	23.18	72.04	39.26	55.65	0.0
	28.30	13.70	21.00	77.62	48.67	63.15	0.0
	23.60	12.94	18.27	76.92	48.97	62.95	0.0
Dec	21.19	8.99	15.09	77.03	48.55	62.79	1.5
	21.31	8.40	14.85	81.87	51.84	66.86	2.6
	18.93	6.32	12.63	87.00	58.13	72.57	0.0
	18.27	7.36	12.82	87.30	57.68	72.49	0.0
Jan	15.92	7.14	11.53	97.14	57.00	77.07	0.0
	16.71	5.40	11.06	92.01	48.69	70.35	21.3
	17.80	7.45	12.62	87.29	52.89	70.09	24.5
	17.14	8.24	12.69	93.96	61.69	77.82	0.0
	19.55	8.78	14.17	89.57	54.50	72.04	16.5
Feb	20.34	12.00	16.17	89.15	58.72	73.93	63.2
	21.44	13.20	17.32	80.81	55.04	67.92	39.5
	21.42	11.42	16.42	73.05	41.84	57.45	0.0
	25.30	13.65	19.50	74.12	45.77	60.05	30.2
March	27.20	15.65	21.42	77.70	45.47	61.58	0.0
	30.55	17.40	23.98	71.20	38.50	54.85	6.7
	31.89	18.11	25.00	62.00	29.00	45.50	0.0
	34.67	20.88	22.77	54.61	29.32	41.96	0.0

Source: Meteorological Data Collection Unit, Department of Physics, Aligarh Muslim University, Aligarh, India.

Appendix 25

Climatic conditions that prevailed during cropping season 2005-06

Months	Temperature			Relative humidity			Rainfall
	Max.	Min.	Mean	Max.	Min.	Mean	
Nov	29.13	18.32	23.72	73.56	43.68	58.62	0.0
	26.92	17.09	22.00	76.22	47.74	61.98	0.0
	26.26	15.60	20.93	72.59	39.66	56.12	0.0
	25.95	13.30	19.63	78.37	47.92	63.15	0.0
	24.40	11.84	18.12	77.37	48.02	62.70	0.7
Dec	23.04	8.44	15.74	76.33	47.90	62.11	0.0
	22.51	7.00	14.75	80.82	51.64	66.23	1.2
	22.18	6.12	14.15	84.50	56.33	70.42	2.1
	20.47	6.36	13.42	88.65	58.68	73.67	0.0
Jan	17.49	5.10	11.29	93.51	57.87	75.69	0.0
	16.75	4.20	10.47	93.16	50.26	72.49	14.6
	21.22	9.00	15.11	87.75	52.17	69.60	0.0
	20.71	7.72	14.21	89.30	56.50	70.30	0.0
	22.92	9.45	16.18	89.09	51.66	68.95	0.0
Feb	24.42	11.92	18.17	90.15	53.70	69.41	0.0
	25.69	12.60	19.14	86.46	55.58	71.28	11.0
	27.74	14.27	21.00	84.47	43.40	64.71	0.0
	26.81	13.35	20.08	83.44	45.19	64.02	0.0
March	28.74	14.37	21.56	78.87	39.54	59.21	3.0
	22.32	17.11	19.72	79.44	55.00	67.22	0.0
	28.95	17.46	23.21	74.15	42.54	58.35	0.0
	31.17	19.33	25.25	69.94	39.87	54.90	0.0

Source: Meteorological Data Collection Unit, Department of Physics, Aligarh Muslim University, Aligarh, India.